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THE CALCIUM, INORGANIC PHOSPHORUS AND MAGNESIUM CONTENT OF THE BLOOD SERUM OF YOUNG HORSES

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The normal ranges of serum calcium, inorganic phosphorus, and magnesium have been reported for mature horses, but such information for young animals is very meager. Pearson ('34) has shown a high negative correlation between age and the inorganic phosphorus content of the serum. The youngest animals studied by him were approximately 6 months of age. The normal range usually given for the calcium and inorganic phosphorus content of the blood serum of mature horses lies between 12 to 13 mg. % and 3 to 4 mg. % respectively. Dimock and Healy ('33) report an average of 12 mg. of calcium and 4.9 mg. of inorganic phosphorus per 100 cc. of blood serum obtained from four thoroughbred yearling fillies. Errington ('37) reports the inorganic phosphorus in the blood sera of ninety-eight horses, 4 years of age and over, and shows a range of 2.56 to 4.83 mg. per 100 cc., mean 3.55 ± 0.045 mg.; for seven colts 2 to 3 years of age, the range is 4.37 to 5.98 mg., mean 5.13 ± 0.226 mg.; eight colts 23 to 43 weeks of age gave a range of 4.02 to 5.78 mg., mean 4.95 ± 0.216 mg. The serum calcium of the same groups of animals, respectively, ranged as follows: 11.50 to 13.70 mg., mean 12.67 ± 0.048 mg.; 12.10 to 13.50 mg., mean 12.95 ± 0.177 mg.; and 11.87 to 14.12 mg.,

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mean 12.81 ± 0.290 mg. Pearson and Catchpole ('36) report normal calcium values of 13.01, 13.59 and 13.11 mg. % for Percherons, Arabians and Shetland ponies, respectively. Eveleth ('37) made analyses of magnesium in the blood and reported a range of between 2 to 3 mg. per 100 cc. of serum.

All the foals born in 1936 and 1937 at the University of California Farm were used as experimental animals in our studies. The 1936 foals ranged from 3 to 6 months of age when the first blood samples were taken in August of that year. Samples were taken at intervals until December, 1937. Blood samples were secured on the day of foaling from both the mare and foal in 1937. Subsequent samples were taken from the foal at intervals until December when the experiment was terminated.

In view of the small number of experimental animals employed and the fact that the values obtained from the chemical analyses overlap and are indistinguishable, it was thought best not to segregate the results either as to breed or sex. However, of the nineteen young animals used, five were Thoroughbreds and the remaining fourteen were Percherons.

Approximately 25 cc. of whole blood were drawn from the jugular vein. The samples were allowed to clot and the clear serum was obtained by centrifugation. The calcium was determined by the Clark-Collip ('25) modification of the Kramer-Tisdall method, and the inorganic phosphorus by the method of Fiske and Subbarow ('25). The magnesium determinations were made according to the method of Briggs ('24).

The mean values of the analyses for each age group, together with their standard errors, are presented in the table. The serum calcium, inorganic phosphorus, and magnesium content of the mares at foaling are within the normal range reported for mature horses. Since the foals present more varied results, each of the constituents will be discussed singly. The magnesium content of the blood serum of the foals is not different from the normal range which has been reported for mature horses. The mean values rise from 2.3 ± 0.10 mg. % at birth to 3.1 ± 0.24 mg. at 9 weeks of age; from then on the

magnesium values are fairly constant hovering around 3.0 mg. per 100 cc. of serum. The greatest difference, 1 mg., is found between the mean value at birth and the mean value for the group of animals ranging from 22 to 25 weeks of age. Apparently the magnesium content of the serum changes little with age. So long as the animal is healthy and receives normal feed the magnesium content of the blood serum remains at a fairly constant level.

TABLE 1

Calcium, inorganic phosphorus, and magnesium content of the blood serum

AGE OF ANIMALS	NO. OF ANIMALS	MILLIGRAMS PER 100 CC. BLOOD SERUM		
		Calcium	Inorganic phosphorus	Magnesium
		Mean	*Mean*	*Mean*
Mare at foaling	10	12.7±0.22	3.3±0.19	2.7±0.18
Birth	10	12.5±0.19	5.1±0.15	2.3±0.10
2 to 4 weeks	9	12.8±0.17	7.7±0.19	2.4±0.11
5 to 7 weeks	6	13.1±0.30	7.6±0.31	2.5±0.06
8 to 10 weeks	3	13.2±0.30	7.9±0.33	3.1±0.24
11 to 13 weeks	5	13.4±0.21	7.4±0.13	2.9±0.11
14 to 16 weeks	4	13.2±0.21	7.4±0.42	3.0±0.16
17 to 19 weeks	3	13.3±0.44	7.1±0.25	3.0±0.22
22 to 25 weeks	4	12.7±0.09	7.0±0.20	3.3±0.13
6 to 12 months	23 [1]	12.2±0.15	5.6±0.09	2.7±0.08 [2]
13 to 16 months	10	13.0±0.23	5.1±0.11	2.9±0.13
19 to 23 months	6	13.5±0.18	4.2±0.08	.. ...
All Ca analyses	83	12.6±0.18	.. ...	.. ...
All Mg analyses	68	... ...	.. ...	2.7±0.05

[1] Some animals were sampled more than once during this period.
[2] Magnesium analyses were made on only fourteen samples.

The calcium content of the serum of the foal at birth is likewise within the normal range for mature horses. Nevertheless there are greater changes in the calcium values than in those for magnesium. The calcium rises from 12.5 ± 0.19 mg. per 100 cc. of serum to 13.4 ± 0.21 mg. per 100 cc. at 12 weeks of age. Despite the fact that this difference, 0.9 mg., is statistically significant, both values are within the normal range which has been reported for mature horses. The mean value 13.5 ± 0.18 mg. per 100 cc. of serum for yearlings 19 to 23

months old is likewise within normal limits as reported by Pearson and Catchpole ('36).

The mean calcium value of 12.2 ± 0.15 mg. per 100 cc. of serum for the 6 to 12 months old foals is of interest. The samples were secured during the winter (December and February) of 1936 and (December) 1937. The figure given in the table represents the mean for samples taken on foals of both years. The low value is due entirely to the samples taken from the 1936 animals. The range for these samples was from 10.7 to 12.9 mg. with a mean of 11.9 ± 0.21 mg. The mean for the 1937 foals was 12.6 ± 0.45 mg. with a range from 12.0 to 13.6 mg. per 100 cc. of serum. The cause of this difference is not known. The mean 12.2 ± 0.15 for the entire group, while within the normal range, approaches some of the values reported by Millen and Eveleth ('38) for horses suffering from encephalomyelitis. The animals used in the present experiment, however, were normal and receiving their normal winter ration. This consisted of alfalfa hay and a grain mixture of oats, barley, and linseed meal. Except for the calcium analyses of the 1936 foals 6 to 12 months of age, all the other calcium analyses fall within the normal range for mature horses. The calcium content of the serum as well as the magnesium exhibits little change with increasing age.

At foaling time the inorganic phosphorus content of the blood serum was significantly higher for the foals than for the mares. The serum of the mares showed a mean inorganic phosphorus content of 3.3 ± 0.19 mg. per 100 cc. which is normal for mature horses. The value for the foals at birth, on the other hand, was 5.1 ± 0.15 mg. %. The analyses showed a rapid rise in the inorganic phosphorus content of the blood serum during the first 2 weeks after birth. This value reached a maximum of 7.9 ± 0.33 mg. % at 8 to 10 weeks of age. The amount of inorganic phosphorus present in the blood serum gradually decreased as the animals became older.

The table shows that the mean inorganic phosphorus values from 2 weeks to 1 year of age are higher than the mean value

for the foals at birth. Moreover, these differences are statistically significant. It is not until the animals are 13 to 16 months of age that the mean inorganic phosphorus value drops to the same level it was at birth. By the time the yearlings are 19 to 23 months old the amount of inorganic phosphorus present in the serum is significantly lower than it was at birth, although it is still higher than for the mares at foaling time.

The amount of inorganic phosphorus reported by Pearson ('34), 5.23 $\pm$ 0.13 mg. per 100 ml. serum, for animals under 1 year of age is similar to the amount, 5.6 $\pm$ 0.09 mg. found in the present data for foals 6 to 12 months old. This latter value is significantly higher than that for the amount of inorganic phosphorus present at birth. The present data in part corroborate Pearson's conclusion that a high negative correlation exists between age and inorganic phosphorus content of the blood serum. The reverse condition, however, is encountered when very young animals (birth to 10 weeks) are considered. In these animals it is seen that the inorganic phosphorus content of the serum increases as the animals approach 10 weeks of age.

SUMMARY

Calcium, inorganic phosphorus, and magnesium analyses of the blood serum of foals ranging from birth to 23 months of age were made. It was found that the amounts of calcium and magnesium present in the serum of these young animals were not different from those that have been reported for adult animals.

The inorganic phosphorus content of the foal's serum was higher at birth than that of the mare. There was a rapid rise during the first 2 weeks after birth in the amount of inorganic phosphorus found in the serum of the foal. A maximum was reached when the foals were about 10 weeks of age. From this point on the amount of inorganic phosphorus gradually decreased as the animals became older.

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CEREALS AND RICKETS

X. THE AVAILABILITY OF PHYTIC ACID PHOSPHORUS [1]

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A study of the interrelations of calcium, phosphorus, and vitamin D in the production of rickets has revealed that not all forms of phosphorus are equally available. For a time the total calcium and phosphorus relations were stressed, but of late the emphasis has shifted to comparisons of the availability of phosphorus in different forms. So far all the investigations bearing on the availability of phytic acid phosphorus, with the exception of that of Jones ('39), have failed to recognize the existence of various forms of phosphorus in cereal grain rations with the result that the data have been inconclusive or conflicting.

The object of the present experiments was to compare the availability of the phosphorus of phytic acid with phosphorus from inorganic sources. These were incorporated in a relatively simple ration, low in phosphorus, but complete in other respects. The resultant diets were fed to young rats and compared for their effect on bone ash, size of the costochondral junctions and the width of metaphyses.

EXPERIMENTAL

The basal ration used was the low phosphorus ration (R-14) of Schneider and Steenbock ('39). Its primary ingredients were cerelose 49.0, egg white 18, starch 20, Vitab 4, cottonseed

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

oil 5, low phosphorus salts 4%, respectively. This ration was supplemented with beta carotene fed in Wesson oil by dropper, 10 micrograms per rat per day. All other additions were made at the expense of the cerelose. The basal ration contained 0.04% phosphorus and 0.57% calcium as determined by analysis. The Ca content of the rations was kept constant at 0.57%, supplied as the carbonate (0.33%) and lactate (0.24%). The only variables in the ration were the kind and amount of phosphorus and the presence or absence of vitamin D.

The phytic acid was prepared from crude calcium phytate[2] according to the method of Boutwell ('17). It was added to the ration as a concentrated solution containing 133.8 mg. phosphorus per cubic centimeter. Of this 123 mg. was phytic acid phosphorus. Of the remainder, 6.34 mg. were shown to be inorganic phosphorus. The total and inorganic phosphorus were determined by the method of Fiske and Subbarow ('25) with an Evelyn photoelectric colorimeter. The phytic acid phosphorus was determined by the technique of McCance and Widdowson ('35). The phytic acid was found to be stable in water solution as no hydrolysis had occurred after 3 months standing although the color of its solution had deepened appreciably. The inorganic supplements were incorporated as an aqueous solution of equimolecular amounts of the mono- and dibasic sodium phosphates. The rations were dried before an electric fan at room temperature after addition of the supplements.

Nicolaysen's ('37) data were used in deciding the level of phosphorus to be fed. He pointed out that a 30 to 60 day old rat required 4.5 mg. phosphorus and 7.5 mg. calcium for each gram increase in body weight. Assuming an optimal rate of growth of 4 gm. per day on a food intake of 7 gm., 0.257 gm. "available" phosphorus and 0.428 gm. calcium were required per 100 gm. of ration. This was accepted as the optimal level of intake. Since some of the rats would not gain 4 gm. daily

[2] For this we are indebted to the E. A. Staley Manufacturing Co. of Decatur, Illinois.

even though they consumed 7 gm. of ration, suboptimal amounts of phosphorus were fed in addition.

In all, seven different rations were fed. These included, besides the optimal amount of phosphorus, levels of one-eighth, one-fourth, and one-half the optimal, plus a negative and positive control. The negative control group received as much inorganic phosphorus as was present as a contaminant in the largest addition of phytic acid. The positive control group received inorganic phosphorus equivalent to the total phosphorus. All the above rations were supplemented with 4.5 U.S.P. units of vitamin D (viosterol) per 100 gm. of ration mixed in ether solution. The ether was volatilized before an electric fan and the ration again mixed thoroughly. One additional ration with phytic acid containing one-fourth of the optimal level of phosphorus without vitamin D was included. Analyses of the rations after 2 weeks standing at room temperature revealed no hydrolysis of the phytic acid. The rations were kept in a refrigerator between feedings to avoid the development of rancidity.

Six 50 to 60 gm. rats were placed on each ration. Litter mates were distributed in the series so that no two rats of the same litter were on the same ration. They were kept individually in screen bottomed cages. They received distilled water ad libitum. A careful record of food consumption was kept; however, no attempts were made to equalize consumption. The rats were weighed and inspected weekly. At the conclusion of the experiment, the rats were anaesthetized and blood was drawn from the abdominal aorta for calcium and phosphorus determinations. The calcium was determined by the method of Clark and Collip ('25) and the phosphorus by the method of Fiske and Subbarow ('25). The radii and ulnae were removed for inspection of metaphyses, and the femora for a determination of ash after extraction with alcohol.

It is evident (table 1) that the inclusion of phytic acid in the low phosphorus ration caused a definite improvement in calcification. This became more evident as the amount of phytic acid phosphorus was increased to the optimal level. There

resulted a definite improvement in food consumption, in body weight, in weight of bone and bone ash, and in the percentage of bone ash. That this improvement was not due to the small amount of contaminating inorganic phosphorus is evident from the negative control (ration XII). The deficiency of phosphorus was also reflected in the blood picture. The average value for serum phosphorus for the negative control was

TABLE 1

The effect of increasing amounts of phytic acid phosphorus on calcification

RATION COMPOSITION (ADDITIONS TO BASAL)	NO. OF RATS	NO. OF WEEKS	FOOD CONS.	CHANGE IN WEIGHT	WEIGHT OF BONE	WEIGHT OF ASH	BONE ASH
			gm.	*gm.*	*gm.*	*gm.*	%
VII—0.032 gm.[1] (⅛ opt.) phytic acid P plus D[2]	5	14	810	73	0.1773	0.0566	32.4
VIII—0.064 gm. (¼ opt.) phytic acid P plus D	6	14	834	95	0.1770	0.0618	35.5
IX—0.128 gm. (½ opt.) phytic acid P plus D	6	14	1045	162	0.2594	0.1228	47.3
X—0.257 gm. (opt.) phytic acid P plus D	4	14	1146	186	0.3075	0.1620	56.3
X ————	2	10	715	135	0.4040	0.2275	52.8
XI—Same as VIII except no vitamin D	6	6	330	39	0.1140	0.0250	22.0
XII—0.0126 gm. inorganic P equal to that in X plus D	5	10	583	63	0.1323	0.0378	28.6
XIII—0.25 gm. (opt.) inorganic P plus D	4	14	1241	208	0.3993	0.2392	61.5
XIII ————	2	10	877	170	0.4615	0.2831	60.0

[1] Per 100 gm.
[2] 4.5 U.S.P. units vitamin D (viosterol) per 100 gm.

3.5 mg. % as compared with values ranging from 7 to 11 mg. % for the other groups. The serum calcium and phosphorus values for the other rats were of the order expected for mildly rachitic to normal rats.

The rats on the one-fourth optimal phosphorus level without vitamin D (ration XI) did so poorly that they were killed at the end of 6 weeks. Similarly the rats in the negative con-

trol group (ration XII) did poorly; they began to lose weight at the end of 10 weeks when they were killed. Since this latter group served as a control for the rats on the optimal phytic acid (ration X) and inorganic (ration XIII) phosphorus levels, representative rats from these two groups were also killed at the end of 10 weeks for comparative purposes. The remainder of the rats were continued for 14 weeks.

The striking difference between the rats on the one-fourth optimal phytic acid level without vitamin D (ration XI) and those receiving vitamin D (ration VIII) indicated the desirability of another experimental series. In this second series one-half optimal phytic acid and inorganic phosphorus with and without vitamin D were fed. As a negative control, inorganic phosphorus equivalent to that in the phytic acid ration was also fed. Four rats were placed on each ration under the same experimental conditions as in the first series except that the experiment was terminated at the end of 9 weeks. This modification was necessitated by the death of some of the rats during the seventh and eighth weeks. The data on the rats which died were not included in the tabulated results.

It is again evident (table 2) that vitamin D played an important role in the utilization of phytic acid phosphorus. Its effect on inorganic phosphorus was less pronounced. A comparison of the rats on one-half optimal inorganic phosphorus without D (ration XXIV) with those on the same ration plus D (ration XXIII) reveals that the former were only mildly rachitic and hence the increment of improvement which could possibly be achieved by the vitamin D was definitely decreased. A comparison of similar rations containing phytic acid phosphorus instead of inorganic phosphorus (rations IX and XXII) reveals that the rats which did not receive vitamin D were markedly rachitic. In fact the percentage of bone ash of the one-half optimal phytic acid phosphorus group without D (ration XXII) approximated the percentage of bone ash from the negative control rations (ration XXV and XXVI). Apparently under these conditions the phosphorus of phytic acid

was not demonstrably available. Vitamin D was without effect in the negative control group.

As no attempts were made to equalize the phosphorus intake of the rats in the different groups, the results suggested the desirability of carrying out a supplementary series of experiments in which this was done. In this the vitamin D additions were increased tenfold and the experiment was terminated at

TABLE 2

Effect of vitamin D on phytic acid and inorganic phosphorus utilization

RATION COMPOSITION (ADDITIONS TO BASAL)	NO. OF RATS	NO. OF WEEKS	FOOD CONS.	CHANGE IN WEIGHT	WEIGHT OF BONE	WEIGHT OF ASH	BONE ASH
			gm.	gm.	gm.	gm.	%
IX—0.128 gm.[1] (½ opt.) phytic acid P plus D[2]	4	9	556	113	0.2013	0.0916	45.4
XXII—Same as IX except no D	2	9	508	66	0.1572	0.0383	24.5
XXIII—0.128 gm. (½ opt.) inorganic P plus D	4	9	683	151	0.3338	0.1681	50.5
XXIV—Same as XXIII except no D	3	9	566	102	0.1440	0.0808	41.9
XXV—0.0063 gm. inorganic P equal to that in IX plus D	4	9	478	60	0.1237	0.0298	24.4
XXVI—Same as XXV except no D	2	9	441	15	0.0984	0.0231	23.5

[1] Per 100 gm.

[2] 4.5 U.S.P. units vitamin D (viosterol) per 100 gm.

the end of 4 weeks. It is evident from the results (table 3) that the presence of vitamin D had no effect on growth. However, vitamin D did have a marked effect on the utilization of phytic acid phosphorus (rations A and B) and a less marked effect on the utilization of inorganic phosphorus (rations C and D). These observations are in agreement with those of the second series. Although the food consumption was almost identical, the rats receiving the inorganic phosphorus, even without vitamin D, were definitely superior to those receiving the same amount of phosphorus as phytic acid with and without vitamin

D. Inspection of the metaphyses and costochondral junctions confirmed the above conclusions which in turn were in direct agreement with the percentage of bone ash. The calcium and phosphorus determinations on the blood sera failed to reveal any anomalies.

TABLE 3

Paired feeding study of the effect of vitamin D on phytic acid and inorganic phosphorus utilization

RATION COMPOSITION (ADDITIONS TO BASAL)	NO. OF RATS	NO. OF WEEKS	FOOD CONS.	CHANGE IN WEIGHT	WEIGHT OF BONE	WEIGHT OF ASH	BONE ASH
			gm.	gm.	gm.	gm.	%
A 0.128 gm.[1] (½ opt.) phytic acid P plus D[2]	6	4	215	39	0.1093	0.0405	37.0
C 0.128 gm. (½ opt.) inorganic P plus D	6	4	215	47	0.1482	0.0654	44.1
B Same as A except no D	6	4	211	39	0.0900	0.0194	21.5
D Same as C except no D	6	4	210	47	0.1280	0.0517	40.4

[1] Per 100 gm.

[2] 45 U.S.P. units vitamin D (viosterol) per 100 gm.

SUMMARY

When rats were fed a cereal free ration of normal calcium content the utilization of phosphorus from phytic acid was markedly enhanced by the addition of vitamin D. This effect was less marked with inorganic phosphorus. In no case was the utilization of phosphorus from phytic acid equal to that of inorganic phosphorus.

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CEREALS AND RICKETS

XI. CALCIUM PHYTATE AS A SOURCE OF CALCIUM[1]

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It is generally assumed that the calcium of compounds ordinarily used to supplement grain rations is entirely available. Steenbock et al. ('23) in their experiments with young rats found the calcium of calcium lactate, carbonate, phosphate, silicate, and sulfate to be available to the rat. Buckner and Martin ('29) observed that calcium carbonate, phosphate, and sulfate served equally well as sources of calcium for the growing chick. Bethke, Kennard and Kick ('29) confirmed the work of Buckner and Martin. They extended their studies to the calcium of limestone, steamed bone meal, rock phosphate, phosphatic limestone, and oyster shells, and found no differences in assimilation. However, Mellanby ('37) is of the opinion that phytic acid actually increases rickets in dogs on moderately rachitogenic diets by the withdrawal of calcium from the body tissues. There is the possibility that even the calcium of calcium phytate taken by mouth might be unavailable. This possibility was accordingly put to test. Mellanby's theory regarding the withdrawal of calcium from the tissues by unhydrolyzed phytic acid will be given consideration in a subsequent paper.

EXPERIMENTAL

The calcium phytate used was prepared from crude calcium phytate[2] according to the method outlined by Anderson ('12).

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] Received from E. A. Staley Manufacturing Co. of Decatur, Ill.

Analyses revealed it to contain 16.9% calcium and 17.0% phosphorus. Of the phosphorus, 0.54% was present in the inorganic form. The basal ration in which it was incorporated was essentially the purified low phosphorus ration of Schneider and Steenbock ('39) with the addition of optimal amounts of mono- and di-potassium phosphates, except for the omission of the calcium salts. The composition of the modified salt mixture was as follows: NaCl 183.7, K_2HPO_4 187.83, KH_2PO_4 147.02, KCl 17.3, $MgSO_4$ 90.0, $MnSO_4$ 0.20, $K_2Al_2(SO_4)_4 \cdot 24H_2O$ 0.09, $CuSO_4 \cdot 5H_2O$ 0.39, NaF 0.57, KI 0.05 and Fe citrate 16.15 parts, by weight, respectively.

Two and fifty-seven hundreds grams were incorporated in each 100 gm. of ration. The basal ration contained 0.019% calcium as determined by the method of Meloche, Clifcorn, and Griem ('33).

The level of calcium to be fed was arrived at on the assumption that the calcium was entirely available (Krieger, Bunkfeldt and Steenbock, '40). Calcium as the carbonate or phytate, to the extent of 0.428 gm., was incorporated in each 100 gm. of ration for the optimal level. The experimental conditions and criteria were the same as those of the previous investigation.

A preliminary experiment of 13 weeks' duration in which calcium phytate was fed at the optimal level and at levels of one-half, one-fourth, and one-eighth of the optimal, revealed that in the presence of vitamin D there was a marked improvement in growth, food consumption, weight of bone, weight of bone ash, and consequently, percentage of bone ash with an increase in the calcium intake up to one-half the optimal level. Little or no difference was observed between the rats in the positive control group receiving calcium carbonate and those receiving the same amount of calcium as calcium phytate. However, when vitamin D was omitted from the calcium phytate ration, calcification was markedly reduced. The percentage of bone ash for the group on one-fourth the optimal calcium intake without vitamin D was 45.3% as compared with 54.8% for the same ration supplemented with vitamin D. As calcium car-

bonate was not fed at an identical level without vitamin D, it appeared desirable to run a second experimental series in which this was done. The experimental conditions in this series were essentially the same as in the first.

It is evident (table 1) that in this series the calcium of calcium phytate was utilized as readily as that of calcium carbonate regardless of whether vitamin D was present or not.

TABLE 1

Comparative study of the availability of calcium in calcium phytate and calcium carbonate

ADDITIONS TO THE BASAL RATION [1]		FOOD CONS.	CHANGE IN WT.	WEIGHT OF BONE	WEIGHT OF ASH	BONE ASH
		grams	*grams*	*grams*	*grams*	%
XIV	0.107 gm. (1/4 opt.) Ca as Ca phytate plus D[2]	728	130	0.2588	0.1314	50.8
XVI	0.107 gm. (1/4 opt.) Ca as $CaCO_3$ plus D	692	127	0.2630	0.1290	49.1
XV	Same as ration XIV except no vitamin D	535	76	0.2005	0.0856	42.5
XVII	Same as ration XVI except no vitamin D	548	58	0.1588	0.0691	43.5
XVIII	0.054 gm. (1/8 opt.) Ca as Ca phytate plus D	663	102	0.2102	0.0878	40.9
XX	0.054 gm. (1/8 opt.) Ca as $CaCO_3$ plus D	582	76	0.1776	0.0730	41.5
XIX	Same as ration XVIII except no vitamin D	502	46	0.1576	0.0579	36.8
XXI	Same as ration XX except no vitamin D	493	53	0.1582	0.0582	35.9

[1] Each ration was fed to six rats for 12 weeks. The additions represent those made for 100 gm. of ration.

[2] 4.5 U.S.P. units vitamin D (viosterol) per 100 gm.

Vitamin D produced a definite increase in growth, food consumption, weight of bone and weight of bone ash and consequently percentage of bone ash at the one-eighth and one-fourth optimal levels. This improvement was probably due to the increased absorption of calcium from the gut as has been demonstrated by Nicolaysen ('37). It was again obvious that the amount of calcium present was the limiting factor

since the higher levels (rations XIV, XV, XVI, and XVII) produced the best growth and calcification. This was also borne out by the blood picture. The serum phosphorus values were normal in all cases, but the calcium values were 3 to 5 mg.% below those obtained at the one-half optimal and optimal levels in the preliminary experiment. The metaphyses and the costochondral junctions revealed normal or almost normal calcification except in those animals which received one-eighth the optimal levels without vitamin D.

SUMMARY

When calcium phytate was added to a cereal-free low-calcium ration, its calcium was found to be as readily available to the rat as the calcium of calcium carbonate. Vitamin D improved the utilization of both forms of calcium to the same extent.

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CALCIUM AND PHOSPHORUS METABOLISM IN RATS AND DOGS AS INFLUENCED BY THE INGESTION OF MINERAL OIL

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Although conclusive data showing the interference of mineral oil with the utilization of vitamin A, both as such and in its precursor form, carotene, have been offered (Burrows and Farr, '27; Dutcher et al., '34; Jorstad and Johnston, '26; Mitchell, '33; Rowntree, '31; Anderson, '39 and Curtis and Kline, '39), the evidence on the effect of mineral oil ingestion upon the availability of vitamn D is conflicting. Much of this evidence has resulted from a search for the best diluent for vitamin D concentrates for use as an antirachitic agent (Dutcher et al., '27; Hawks et al., '29; Jackson, '31, '34 a, 34 b).

The experiments presented in this paper were designed to gain further information regarding the influence of mineral oil ingestion by rats and also by dogs upon the metabolism of calcium and phosphorus in these species. In rats, the effect of continuous ingestion of mineral oil upon calcium and phosphorus metabolism has been studied by comparing the degree of calcification of rachitic lesions (as shown by the "line test") afforded by various levels of vitamin D fed as cod liver oil in the presence and absence of mineral oil. In dogs, continuous calcium and phosphorus "balance studies" were conducted for 11 weeks.

EXPERIMENTAL

Amount and method of administration of mineral oil

In order that the results of this work might have practical significance, consideration of the therapeutic dose for humans was made in the selection of the amount of mineral oil to be fed to the experimental animals. The difficulty, however, of arriving at a comparable therapeutic dose for rats is apparent when the differences in size, food intake, eating habits and rate of growth and development are realized.

In the experiments described herein, the mineral oil [1] was incorporated in the basal ration at two different percentage levels, i.e., 5 and 10%. In this way, the amount of mineral oil ingested was automatically proportional to the food or calorie intake (Jackson, '31), and lubrication was continuous. The fat soluble vitamin supplements were fed separately (Jackson, '34 a). Food consumption records showed an average daily intake of mineral oil by the rats of from 0.2 to 0.5 cc.

Jackson ('31) and Rowntree ('31) have independently estimated that 0.5 cc. of mineral oil daily per rat was comparable to an average human therapeutic dose of 30 cc. This amount of mineral oil was found to cause no diarrhea. The feces were bulkier and softer but preliminary tests using carmine as a marker, showed that even the higher level of mineral oil ingestion produced slight, if any, acceleration in the rate of elimination (Smith and Spector, '40).

EXPERIMENTS WITH RATS

Young rats weighing from 55 to 65 gm. were placed upon Steenbock's high-calcium low-phosphorus rickets-producing ration no. 2965 which is composed of ground yellow corn 76, wheat gluten 20, calcium carbonate 3, and sodium chloride 1%, respectively. When care was taken to keep the rats in a dark room from which all direct light was excluded, the animals regularly developed severe rickets in 21 days as indicated by

[1] The mineral oil used was a heavy California liquid petrolatum product of E. R. Squibb and Sons.

the findings of the "line test" developed by McCollum and his co-workers ('22). At this time, two or more representative rats from each litter were continued as controls upon the rickets-producing ration no. 2965 whereas the remainder of the litter were given the same ration in which mineral oil was incorporated. Each rat was housed individually in a round metal cage with a raised screen bottom and given the basal diet and distilled water ad libitum.

In addition to the basal ration, each rat received a daily feeding of cod liver oil which was introduced by pipette directly into its mouth. No difference in the results obtained was found when the vitamin D supplement was given at 8 A.M. or 6 P.M. after a day of fast. The cod liver oil was so diluted with corn oil that 0.05 cc. fed daily during a 10-day test period produced a continuous line of calcification at the zone of provisional calcification in the rachitic metaphyses of the control animals. Using the Bills et al. ('31) system for grading the degree of healing of the rachitic lesions, it may be seen in table 1 that an average of 2.4+ healing was recorded for the thirty-six rats receiving 0.05 cc. of the cod liver oil solution.

Graded amounts of this same oil dilution were given to the litter mate mineral oil-fed rats and the degree of calcification of the rachitic lesions after a 10-day feeding period was again judged according to the same standards.

The data which are summarized in table 1 plainly show that an amount of cod liver oil, which produced a continuous line of calcification in the rachitic metaphyses of the rats receiving the basal diet 2965 alone, produced no healing whatsoever when mineral oil incorporated in the same ration was ingested. When the amount of cod liver oil fed was increased, some healing occurred, but it may be seen that at least three times as much vitamin D as cod liver oil was necessary to produce the same degree of calcification when 5% mineral oil was present in the ration. Again, between five and ten times as much cod liver oil was necessary to compensate for the ingestion of mineral oil at the higher level. Thus, proof

was afforded of an interference with the utilization of the vitamin D in cod liver oil, and therefore of a decrease in the antirachitic potency of cod liver oil resulting from the ingestion of mineral oil. Records of the food consumption of these rats showed an average food intake of slightly less than 50 gm. during the test period. It is evident, therefore, that the amount of mineral oil ingested even at the higher level of incorporation in the ration was not excessive and was probably less than a comparable therapeutic dose for humans.

TABLE 1

Summarized "line test" findings showing the effect of mineral oil ingestion upon the calcification of rachitic lesions induced by cod liver oil

AMOUNT OF COD LIVER OIL FED	DIET 2965 ALONE			DIET 2965 PLUS 5% MINERAL OIL			DIET 2965 PLUS 10% MINERAL OIL		
	Number of rats	Average food intake	Average degree of healing [1]	Number of rats	Average food intake	Average degree of healing [1]	Number of rats	Average food intake	Average degree of healing [1]
cc.		*gm.*			*gm.*			*gm.*	
0.05	36	48	24+	11	46	0.0	12	47	0.0
0.1				12	48	1.8+	7	43	0.0
0.10				9	46	2.4+	13	45	1.6+
0.25				4	49	2.8+	13	47	2.0+
0.5				3'	48	4.0+	3	48	2.8+

[1] Bills et al. ('31).

EXPERIMENT WITH DOGS

Although most workers have used rats for the study of factors affecting the production of rickets and especially for vitamin D assays, Mellanby ('19) has used dogs for the study of experimental rickets, believing that the course of rickets in puppies more closely resembles that in human beings. In dogs, as in children, a deficiency of vitamin D alone is sufficient to produce rickets without any distortion of the diet in other respects, whereas in rats, the calcium and phosphorus relationship in the diet must be abnormal as well.

Accordingly, a study has been made of the effect of mineral oil ingestion by puppies receiving a ration probably optimum in calcium and phosphorus content and adequately provided with vitamin D. In this phase of the investigation, the effect

of mineral oil ingestion has been observed by means of continuous calcium and phosphorus "balance experiments" for an 11-week period during the ages of very rapid growth and development.

Methods and materials

Four mongrel puppies (Scottie mother) weaned at 4 weeks of age were given 47.5 gm. per kilo of a ration similar to Morgan and Garrison's ('30) modification of the Karr-Cowgill laboratory ration for dogs. The constituents of this ration were thoroughly mixed and then thinned with distilled water to a mushy consistency at the time of feeding. The diet was supplemented by the daily feeding of 400 U.S.P. units of vitamin A [2] per kilo. Previous findings of this laboratory (Bradfield and Smith, '38) showed that normal growth of puppies was provided for, and a slight storage of vitamin A in the liver was permitted, when half this amount of vitamin A was administered daily. At this level of ingestion, carotene as a source of vitamin A appeared to be equally as well utilized as cod liver oil.

Vitamin D was fed separately as cod liver oil to all the dogs, except the negative control animal no. 35, in the amount of 1 U.S.P. unit per kilo of body weight per day (Arnold and Elvehjem, '37).

When the puppies had become accustomed to their basal ration and were observed to be developing normally, they were housed in individual wire mesh metabolism cages designed to permit collection of both the urine and feces. The cages were protected against strong sunlight by enclosing them in heavy brown paper and keeping them in the laboratory after the experimental regime was begun.

Dog 35 was placed on the vitamin A supplemented basal ration without vitamin D, thus serving as the negative control. Dog 36 was given the basal ration with both vitamin A and D supplements fed separately and served as the positive control. Dog 37 was given the basal ration in which 5%

[2] Given as S M A C O, a commercial solution of carotene in oil.

mineral oil was incorporated, and received vitamins A and D fed separately. Dog 38 subsisted on the basal ration in which 10% mineral oil was incorporated, with vitamin A and D supplements fed separately in the same amounts as given to the other dogs.

All of the puppies received distilled water ad libitum. They were fed once a day in the morning and the vitamin supplements were given in the late afternoon. In most cases the food was quickly consumed but in instances in which part of it was refused after allowing a reasonable period for its consumption, it was returned to the refrigerator and fed the next day before a fresh allotment was given. A record of the food consumed by each dog was kept for the duration of the experimental period.

The feces and urine were collected daily, and at the end of each 7-day balance period each cage was washed with distilled water and the wash water added to the total collection for the week. Aliquots of the homogenous mixture were analyzed for calcium by the method of McCrudden ('12) and for phosphorus by the method of Fiske and Subbarow ('25).

Samples of the basal ration were analyzed by the same methods and these analyses in combination with the food consumption records gave the basis for data concerning the calcium and phosphorus intakes of the dogs during each 7-day balance period.

Results

Summarized results of the calcium and phosphorus balance studies in which dogs were used are presented in table 2.

It may be seen that in every balance period, the mineral oil fed dogs retained less calcium and phosphorus than did the control animal which received no mineral oil, whether the results are expressed in terms of the absolute amount of these elements retained, or the amount retained per kilo of body weight, or the percentage of the ingested calcium and phosphorus which was retained in the body. In most balance

TABLE 2

The effect of mineral oil ingestion upon the metabolism of calcium and phosphorus in dogs

SERIES AND PERIOD	DOG NO.	CALCIUM RETENTION			PHOSPHORUS RETENTION		
		Total per day	Per cent of intake	Per kilogram per day	Total per day	Per cent of intake	Per kilogram per day
		gm.		*gm.*	*gm.*		*gm.*
A-1 [1] Age 65–71 days	35 Negative control	0.85	23.8	0.0224	0.49	11.3	0.0108
	36 Positive control	1.94	71.1	0.1379	1.17	35.0	0.0422
	37 5% mineral oil						
	38 10% mineral oil	1.16	61.2	0.0396	0.60	24.5	0.0204
A-2 Age 72–75 days	35 Negative control	2.73	52.2	0.0674	2.00	31.5	0.0495
	36 Positive control	4.10	84.1	0.1379	2.91	48.9	0.0977
	37 5% mineral oil	2.95	69.0	0.1094	4.14	79.3	0.1534
	38 10% mineral oil	2.18	61.8	0.0736	2.06	47.9	0.0695
A-3 Age 79–85 days	35 Negative control	4.50	59.7	0.1003	4.12	44.8	0.0919
	36 Positive control	5.63	88.5	0.1550	5.94	75.5	0.1635
A-4 Age 86–92 days	35 Negative control	3.16	52.5	0.0622	2.71	36.8	0.0533
	36 Positive control	5.64	85.9	0.1365	2.41	30.1	0.0583
	37 5% mineral oil	1.95	50.3	0.0524	1.23	26.1	0.0301
	38 10% mineral oil	1.76	44.3	0.0492	1.26	26.0	0.0353
A-5 Age 93–99 days	35 Negative control	4.63	55.7	0.0682	4.02	39.7	0.0750
	36 Positive control	6.77	85.1	0.1470	6.64	68.4	0.1443
	37 5% mineral oil	1.33	57.4	0.0372	0.63	22.3	0.0176
	38 10% mineral oil	1.98	51.7	0.0525	1.71	36.7	0.0454
A-6 Age 100–106 days	35 Negative control	3.71	47.4	0.0588	2.21	23.1	0.0350
	36 Positive control	6.61	90.4	0.1215	4.95	55.6	0.0919
	37 5% mineral oil	1.01	44.0	0.0268	0.28	10.2	0.0075
	38 10% mineral oil	1.09	38.0	0.0270	0.80	22.8	0.0197

TABLE 2—*Continued*

SERIES AND PERIOD	DOG NO.	CALCIUM RETENTION			PHOSPHORUS RETENTION		
		Total per day	Per cent of intake	Per kilogram per day	Total per day	Per cent of intake	Per kilogram per day
		gm.		gm.	gm.		gm.
B-1[2] Age 107–113 days	35 Negative control	6.11	65.2	0.0953	4.85	42.5	0.0968
	36 Positive control	7.49	95.9	0.1309	6.30	66.2	0.1102
	37 5% mineral oil	3.61	71.2	0.0948	3.36	54.4	0.0882
	38 10% mineral oil	1.87	53.1	0.0461	1.95	45.4	0.0482
B-2 Age 114–120 days	35 Negative control	8.67	94.4	0.1221	6.72	60.0	0.0924
	36 Positive control	7.54	93.9	0.1144	5.75	58.7	0.0874
	37 5% mineral oil	5.58	79.4	0.1244	4.78	55.7	0.1065
	38 10% mineral oil	3.65	69.3	0.0821	3.14	48.9	0.0707
B-3 Age 121–127 days	35 Negative control	8.62	95.2	0.1180	5.96	53.9	0.0817
	36 Positive control	8.34	95.4	0.1251	6.03	56.5	0.0904
	37 5% mineral oil	7.77	94.9	0.1491	5.71	57.1	0.1161
	38 10% mineral oil	5.42	71.9	0.1171	4.86	53.0	0.1053
B-4 Age 128–134 days	35 Negative control	8.42	93.2	0.1058	7.81	70.8	0.0984
	36 Positive control	7.93	95.1	0.1056	7.11	69.9	0.0946
	37 5% mineral oil	6.62	79.9	0.1150	5.96	59.0	0.1035
	38 10% mineral oil	....	...	.....	....	...	.....
B-5 Age 135–141 days	35 Negative control	10.71	95.0	0.1252	10.80	78.6	0.1263
	36 Positive control	9.03	94.5	0.1146	8.90	76.3	0.1129
	37 5% mineral oil	5.88	73.0	0.0949	6.30	64.2	0.1018
	38 10% mineral oil	3.81	51.8	0.0671	5.15	57.4	0.0908

[1] In balance series A, all dogs were fed 1 U.S.P. unit of vitamin D per kilogram per day except negative control no. 35.

[2] In balance series B, all dogs were fed 5 U.S.P. units of vitamin D per day including the negative control no. 35.

periods the dogs receiving the mineral oil retained even less calcium and phosphorus than did the negative control dog which was given no cod liver oil supplement of vitamin D. For example, in the first balance study of 6 weeks' duration, during which time the dogs received 1 U.S.P. unit of vitamin D per kilo of body weight daily, the mineral oil fed puppies retained only about a third as much calcium and phosphorus as did the positive control dog given the same basal ration and the same amount of the vitamin D supplement. Expressed in relation to body weight, the positive control dog each day retained an average of 0.0181 gm. of calcium and 0.0142 gm. of phosphorus per kilo as compared with an average daily retention of 0.0069 gm. of calcium and 0.0054 gm. of phosphorus by the litter mate dog whose ration contained 10% mineral oil. Thus, in spite of supposedly adequate intakes of both calcium and phosphorus and of the vitamin which regulates the metabolism of these mineral elements, the mineral oil fed dogs were not able to make satisfactory use of these essential dietary constituents probably because of the interference with the utilization of vitamin D as a result of the mineral oil ingestion.

This inability was also clearly reflected in the general nutritive condition of the puppies. The characteristic clinical evidences of severe rickets were apparent in the negative control dog which was given no vitamin D, and even more severe symptoms were noted in the mineral oil fed puppies which received 1 U.S.P. unit of vitamin D per kilo of body weight daily in the form of cod liver oil. These puppies showed the flat footed condition, bowing of the forelegs, rachitic posture of the hind legs, swelling of the wrist and ankle joints, general weakness and inability to support the weight of the body, which are the characteristics of severe rickets. In addition they became very timid, displayed signs of pain and developed thin straggly hair, the latter characteristic also of vitamin A deficiency. The negative control dog was decidedly bow-legged but was more active and had a thicker, better appearing coat of fur than did its mineral oil fed litter mates.

In the second series of balance studies on the same dogs, the level of feeding of vitamin D was increased fivefold, i.e., 5 U.S.P. units per kilo were given daily. In order to have a basis for comparison of the curative effect of this increased dosage in the presence and absence of mineral oil in the ration, the negative control rachitic dog no. 35 was given the same relatively high dose of cod liver oil as the mineral oil fed dogs and their comparative metabolism of calcium and phosphorus observed as before. The results are summarized as balance series B in table 2.

When vitamin D as cod liver oil was given to dog 35 which previously served as the negative control, the retention of both calcium and phosphorus immediately increased. The average daily retention of calcium increased from 0.0094 gm. per kilo in the first period when no cod liver oil was given to 0.0162 gm. in the second period. The percentage of ingested calcium which was retained increased from 45 to 88%, and there were accompanying parallel increases in the retention of phosphorus. Dog 35 was retaining therefore, practically as much of these mineral elements as dog 36 which had always served as the positive control. These changes were reflected in the outward characteristics of dog 35 which changed from an inactive, unsteady, timid animal with severely bowed forelegs to a lively dog which appeared normal except for the enlarged wrist and ankle joints, evidences of his previous rachitic condition. Thus, ample evidence was afforded that the basal ration was rendered complete by the addition of vitamin D as cod liver oil.

Again it may be noted, however, that the retentions of calcium and phosphorus by the mineral oil fed dogs were not optimal. This was especially true in the case of dog no. 38 which received the basal ration containing 10% mineral oil. Increasing fivefold the amount of cod liver oil fed to this dog, although resulting in a greatly increased retention of the bone forming minerals, and a decided improvement in the nutritive condition of the animal, did not lead to optimal retention. Dog 38 in the second period retained somewhat less than two-thirds the amount of calcium and phosphorus

retained by the rachitic negative control dog which was given the same dosage of vitamin D. Here again, as in the case of the rachitic rats previously discussed, the apparent potency of cod liver oil in the healing of rachitic lesions was diminished by the presence of mineral oil in the alimentary canal.

SUMMARY AND CONCLUSIONS

The effect of mineral oil ingestion on the metabolism of calcium and phosphorus has been studied in rats by comparing the ability of vitamin D fed as cod liver oil, to calcify rachitic lesions in the presence and absence of mineral oil, and in dogs, by comparing the retention of calcium and phosphorus by means of a series of weekly balance studies in mineral oil and non-mineral oil fed puppies. The following observations were made:

1. Mineral oil ingestion interfered with the utilization of vitamin D fed separately as cod liver oil to such an extent that three times as much cod liver oil was necessary to induce healing of the rachitic lesions of rats when the basal ration contained 5% mineral oil, and somewhere between five and ten times as much was needed when 10% mineral oil was incorporated in the basal ration.

2. The balance studies showed that mineral oil ingestion by young dogs interfered with the retention of both calcium and phosphorus so seriously that normal calcification of the bony structure was not possible, the mineral oil fed dogs showing the characteristics of severe rickets even though they received adequate amounts of calcium and phosphorus and were given a supposedly minimum protective dose of cod liver oil. Increasing the amount of cod liver oil fivefold did not provide for optimal retention of the mineral elements in the dog receiving the ration containing 10% mineral oil.

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TASTE THRESHOLDS AND TASTE PREFERENCES OF RATS FOR FIVE COMMON SUGARS [1]

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FIVE FIGURES

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In previous experiments taste thresholds for salt and sucrose were determined for rats and humans. Taste thresholds have now been determined for other sugars—maltose, glucose, galactose, and lactose. In addition, a study was made of the taste preferences for these five sugars. The present study deals with the results obtained with rats; the results of a similar study made on humans will be reported later.

Before presenting these results a brief account of our previous experiments will help to orient our interest in taste thresholds. Until recently taste studies were purely of academic interest with little or no relationship to problems of nutrition. The results of our observations have shown that taste may play an important part as a guide to nutritional needs.

Our studies on self-selection of diets are based on the concept of the internal environment first advanced by Claude Bernard (1859). Bernard stated then that, "It is the fixity of the 'milieu interne' which is the condition of free and independent life; and all the vital mechanisms, however varied they may be, have only one object, that of preserving

[1] This work was supported in part by a grant from the Committee for Research in Endocrinology of the National Research Council.

constant the condition of life in the internal environment." Bernard, and later Cannon, who greatly extended this concept ('32), concerned themselves with the physiological mechanisms which stabilize the internal environment, such as, for instance, changes in pulse rate, blood pressure, fat metabolism, etc.

In our studies we have found that behavior factors, or responses of the total organism, may also contribute to this end. One example will suffice to illustrate what is meant by behavior factors. In the normal organism the regulation of the sodium balance depends on the secretions from the cortex of the adrenal glands (Loeb, '33; Harrop et al., '33). After removal of these glands, the body excretes large amounts of sodium in the form of the chloride salt, and the internal environment becomes changed to such an extent that the organism cannot survive; the rats develop symptoms of insufficiency and die in 10 to 20 days. Administration of sodium chloride, either by injection or by feeding, will keep the adrenalectomized rats alive. We were interested to know whether, if given free access to salt in a container separate from the food, adrenalectomized rats would of their own accord take a sufficient amount of salt to maintain a normal internal salt balance and thus keep themselves alive. For this purpose rats were kept in cages which contained a food cup and two graduated bottles, one filled with a 3% solution of sodium chloride, the other with tap water. Records were made daily of the fluid intake. After base lines were obtained of the fluid intake, we removed the adrenals. Very shortly after operation, in some instances even on the same day, the rats began to drink more salt solution (Richter, '36). After 30 to 40 days their daily intake averaged five to twenty times above their preoperative level. The rats survived and gained weight. Thus, after the internal or physiological means of regulation of the sodium balance had ceased to function, the animals themselves took care of the balance by ingesting large amounts of salt.

We do not know the relative importance of the roles played by the physiological and behavior factors in the normal organism. The experiments in this laboratory have shown that these behavior factors participate in a wide range of functions, regulation of calcium metabolism after parathyroidectomy (Richter and Eckert, '37), fat and carbohydrate metabolism after pancreatectomy (Richter and Schmidt, '40), and similar metabolic disturbances in vitamin B deficiency (Richter and Barelare, '39). The rats show normal growth, reproduction, and activity on diets which they select from purified substances—minerals, crystallized vitamins, purified fats, carbohydrates, and proteins (Richter, Holt and Barelare, '38).

Having demonstrated that rats make beneficial dietary selections from purified substances, little doubt remained that natural foods must also be selected by them on the basis of taste. With natural foods the situation is more complicated because of the many different ingredients—minerals, vitamins, etc. When an animal or a human craves a certain food, we do not know which ingredient or ingredients he wants. We could narrow down the field if we knew which of the ingredients were present in a sufficiently large amount to be tasted, that is, in concentrations above the taste threshold.

To supply this knowledge the taste thresholds of rats and humans for many different substances, both beneficial and harmful, have been determined. These studies have opened up an entirely new approach to the problem of the relation of the taste of substances to their nutritional value.

The technique which was used for the experiments on rats is quite simple. The cages were of the same type as in the adrenalectomy and salt appetite experiments. Each cage contained two graduated inverted bottles. In these experiments distilled water was put in both bottles for the first 10 to 15 days. At the end of this time the daily intake from each bottle had usually reached a fairly constant amount. Then one bottle was filled with a very low concentration of the solution to be tested, and thereafter the strength of the solution was increased daily in small steps. We have found so

far that with substances which are needed for normal nutrition, such as sodium chloride, sodium phosphate, potassium chloride, and sucrose, when the concentrations reach a certain level, the rats will begin drinking more of the solution and less of the water, while with harmful or poisonous solutions, such as those of mercuric chloride and arsenic trioxide, when the concentrations reach a certain point, the rats take less of the solution and more of the water. The point at which the rats first indicated that they recognized a difference between the distilled water and the solutions was taken as the taste threshold.

Studies made on adrenalectomized rats bring out the significance and possible practical uses of taste threshold investigations. Their salt threshold fell far below normal. They distinguished between salt solution and distilled water in concentrations of 0.003%, or 1 part of salt to 33,000 parts of water, while normal rats did not make this distinction until a solution of 0.055%, or 1 part of salt to 2000 parts of water, had been reached (Richter, '39).

Studies which were made on humans with a comparable choice technique between a solution and distilled water showed that humans and rats have almost the same salt thresholds. Humans distinguished salt solution from distilled water in concentrations of 0.016%, but did not recognize a salt taste until the concentration reached 0.087%, which is only slightly above the concentration at which rats showed their preference for salt solution (Richter and MacLean, '39).

As yet we have not made any studies on patients with Addison's disease or with other diseases involving destruction of the adrenal cortex. We do know, however, that such individuals, like the adrenalectomized rats, often show a very strong craving for salt (Wilkins and Richter, '40).

Similar studies were made on sucrose taste thresholds. Rats first distinguished between distilled water and sucrose solution in concentrations averaging 0.5%. Studies which were made on humans again showed very close agreement with the animal experiments. Humans first recognized a difference

between sucrose solution and distilled water in concentrations averaging 0.1%, but did not recognize a sweet taste until the concentration reached 0.41% (Richter and Campbell, '40).

In the following experiments a study was made of the rat's responses to five common sugars—maltose, glucose, sucrose, galactose, and lactose. We were interested to determine if, when the rats first distinguished between solutions of these sugars and water, they would indicate a preference or a dislike for the above carbohydrates. Also a comparison of the thresholds of these different sugars was made.

In these experiments, after the thresholds had been reached, the concentrations of the solutions were increased daily in large steps up to the point of saturation for maltose, galactose,

TABLE 1

Basal diet [1]

	gm.
Graham flour	72.5
Casein	10.0
Skim milk powder	10.0
Butter	5.0
Calcium carbonate	1.5
Sodium chloride	1.0

[1] Suggested by Dr. E. V. McCollum and used in this laboratory as a standard stock diet for all laboratory rats.

and lactose and up to 80% for glucose and sucrose. We were interested to learn how much of each of the sugars the rats take at the different concentrations, and whether they would show a preference for each of the sugars at any particular concentration.

METHODS

Previous papers contain a full description of the technique used for taste threshold experiments in rats. The rats were kept in separate cages, each of which contained two graduated inverted 100 cc. bottles and a food cup filled with a mixture made according to the formula in table 1. Fat constituted 14.1%, carbohydrate, 59.2%, and protein, 26.7% of this food mixture.

The intake from the two bottles was measured daily; body weight was recorded weekly.

For 10 to 20 days distilled water was offered in both bottles. At the end of this time, when usually the daily intake from each bottle had reached a fairly constant amount, one bottle was filled with a subliminal concentration of a sugar solution. Thereafter the concentration was increased daily in small steps until the rats definitely distinguished between the sugar solution and distilled water. From then on we increased the sugar strength in much larger steps. Table 2 gives the concentrations used in the glucose experiments and may serve to explain the time-concentration abscissae of the diagrams.

TABLE 2

Sugar concentrations used in the experiments

	%	%	%	%	%
	0.01	0.50	1.80	11.0	40.0
	0.02	0.60	2.00	13.0	45.0
	0.04	0.70	2.20	15.0	50.0
	0.06	0.80	2.40	17.0	55.0
Glucose	0.08	0.90	2.80	19.0	60.0
	0.10	1.00	3.00	21.0	65.0
	0.20	1.20	5.00	25.0	70.0
	0.30	1.40	7.00	30.0	75.0
	0.40	1.60	9.00	35.0	80.0
Maltose	From 0.01 to 50% in 24 steps				
Sucrose	From 0.20 to 80% in 28 steps				
Galactose	From 0.01 to 25% in 35 steps				
Lactose	From 0.01 to 17% in 35 steps				

The concentrations of the other sugars roughly followed this same progression up to 80% or up to their respective saturation points.

Throughout the entire experiment each rat kept its own two bottles which were marked with adhesive tape. Each bottle remained in one particular corner of the cage.

Further, in order to eliminate as far as possible any difference between the distilled water and the sugar solution, other than taste, we took the distilled water for the water bottles and the sugar solutions from the same source of

supply. The sugar solutions were watched closely for gross bacterial and fungoid contamination; and in all instances of contamination the bottles were washed with soap before autoclaving or cleaned with potassium dichromate cleaning solution when an adherent film was present. We prepared fresh sugar solutions daily.[2]

Heating in a 50°C. water bath for 15 minutes dissolved the sugars and also stabilized the mutarotations, thus avoiding differences in taste which might depend on the alpha and beta forms. The solutions were not used until they had cooled to room temperature.

Forty-four young adult male rats of approximately the same age, 65 days, were used in these experiments.

RESULTS

Preference between water and sugar solutions

At the threshold concentration the rats showed a definite preference for maltose and glucose over distilled water, just as they had shown for sucrose. Figure 1 gives the average daily intake of distilled water and glucose solution for eight rats. The ordinates give the fluid intake in cubic centimeters; the abscissae, time in days and also the concentrations of the glucose solution which were offered each day. The first 10 days give the intake records of the distilled water from the two bottles. During this time the intake remained fairly constant and approximately the same for each bottle. Seven days after the rats had a choice between glucose solution and distilled water, they began drinking more glucose solution and less water, evidently because at this time the strength of the glucose solution had reached the taste threshold. Each day thereafter, until the concentration reached 11%, the intake of the glucose solution increased at a rapid rate. As the concentration increased beyond this point, the intake of the glucose solution decreased sharply. Figures 2A and 2B show

[2] The sucrose (U.S.P.), glucose (U.S.P.) and lactose (U.S.P.) were obtained from Merck and Company, Inc.; the maltose (C.P.) and galactose (C.P.) from the Pfanstiehl Company. Due to the high cost of purified levulose, we could not use this sugar in the present study.

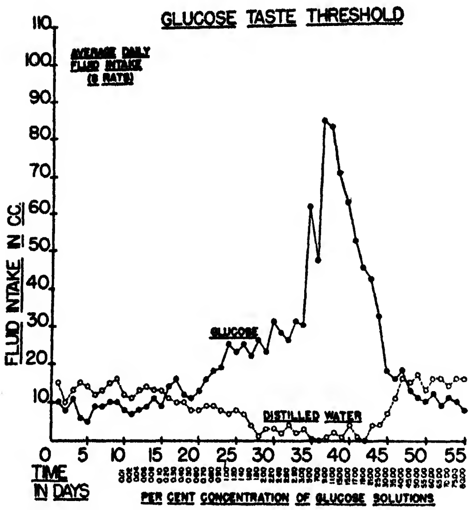

Fig. 1 Curves showing the average daily intake of distilled water and glucose solution of varying concentrations for eight rats. The ordinates give fluid intake in cubic centimeters; the abscissae, time in days and the concentrations of the glucose solutions in per cent.

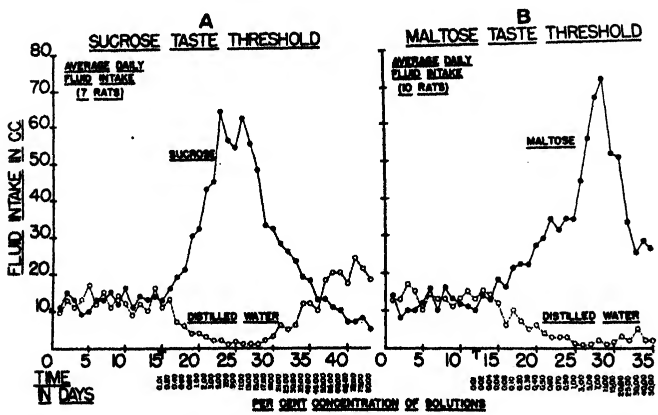

Fig. 2A Curves showing the average daily intake of distilled water and sucrose solution for seven rats.

Fig. 2B Same for maltose solution (ten rats).

the average curves for sucrose (seven rats) and maltose (ten rats) which were similar to those of glucose.

The rats showed only a mild preference for galactose over distilled water. Figure 3 gives the average curve for ten rats. The intake reached a less well-defined peak and then decreased sharply.

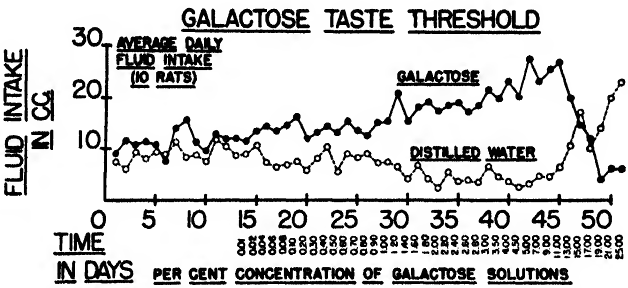

Fig. 3 Curves showing the average daily intake of distilled water and galactose solution for ten rats.

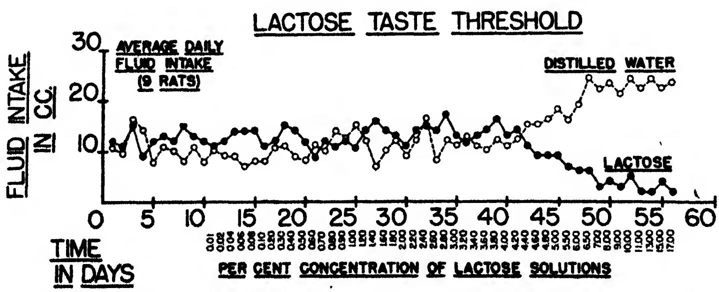

Fig. 4 Curves showing the average daily intake of distilled water and lactose solution for nine rats.

In marked contrast to the rats on the above carbohydrates, nine rats did not definitely prefer lactose to distilled water. Four rats showed a doubtful slight temporary preference (table 1). Figure 4 shows that the average intake curves for the two bottles remained almost the same up to the forty-first day, when the concentration had reached 4.8%, at which point the rats drank less of the lactose solution than of the distilled water.

Taste thresholds

The concentration at which the rats began drinking more sugar solution and less distilled water, and which we have taken as the taste threshold, was far lower for maltose than for any of the other sugars. Table 3 summarizes the results. It gives the thresholds for the individual rats and the average for each sugar. The thresholds of the ten rats on maltose averaged 0.06% and ranged from 0.04 to 0.08%. The eight

TABLE 3

Taste thresholds in per cent

MALTOSE		GLUCOSE		SUCROSE		GALACTOSE		LACTOSE		
Rat	Taste	Rat	Taste	Rat	Taste	Rat	Taste	Rat	Taste (Incr.)	Taste (Decr.)
1	0.08	1	0.2	1	0.8	1	1.8	1	...	2.8
2	0.04	2	0.1	2	0.4	2	1.8	2	1.6?	6.0
3	0.06	3	0.3	3	0.4	3	2.8	3	2.2?	5.0
4	0.08	4	0.3	4	0.8	4	1.2	4	...	4.6
5	0.06	5	0.4	5	0.1	5	1.8	5	2.2?	4.2
6	0.04	6	0.2	6	0.6	6	1.6	6	...	6.5
7	0.08	7	0.3	7	0.4	7	2.4	7	2.4?	6.5
8	0.06	8	0.2			8	0.5	8	...	3.0
9	0.06					9	1.0	9	...	4.6
10	0.08					10	1.2			
Average	0.06		0.20		0.57		1.6		2.2?	4.8
Variation	0.04–0.08		0.1–0.4		0.1–0.8		0.5–2.8		...	2.8–6.5

rats on glucose had the next lowest threshold, averaging 0.20% and ranging from 0.1 to 0.4%. Next came the threshold of the sucrose rats, averaging 0.57% and ranging from 0.1 to 0.8%; next, the threshold of the galactose rats, averaging 1.6% and ranging from 0.5 to 2.8%. Four of the nine rats on lactose increased their intake very slightly at the levels indicated on the table. All nine rats decreased their lactose intake very definitely at an average of 4.8%, and a range of variation from 2.8 to 6.5%. The points at which the sugar and distilled water curves diverged may be seen in the respective charts.

A comparison of the thresholds of these sugars, using maltose as 1, gives the group relationships indicated in table 4. We have not been able to correlate these differences with physical and chemical properties of these sugars.

TABLE 4

Taste thresholds taking maltose as unity

	%	RATING
Maltose	0.06	1
Glucose	0.20	5
Sucrose	0.57	10
Galactose	1.60	30
Lactose	2.20 (?)......	40 (?)
	4.2	70

Maximum preference concentrations

The concentration at which the rats drank the largest amounts of the different sugar solutions varied less than did the taste thresholds. Table 5 summarizes the results:

TABLE 5

Maximum preference concentrations

SUGAR	PEAK CONCENTRATION	AVERAGE DAILY INTAKE OF SUGAR AT PEAK CONCENTRATION
	%	cc.
Glucose	11 (9–13)	90 (73–103)
Maltose	10 (7–11)	75 (61– 91)
Sucrose	8 (5–13)	65 (50–119)
Galactose	9 (5–13)	28 (22– 49)
Lactose	....	15 (highest daily average at any time)

Thus, for the four sugars—glucose, maltose, sucrose and galactose—the peak concentration averaged 8.5% and ranged from 8 to 11%. The amount of the solution taken daily at the peak concentration varied through a wider range, from 90 cc. for glucose solution to 28 cc. for galactose, or to 15 cc. for the highest average reached for lactose at any time.

In considering the significance of this peak concentration, we must take into account the fact that at the peak the rats on glucose, maltose, and sucrose received with the solution from three to four times as much water as normally. It is quite possible that an excess supply of water may have stopped the rats from drinking even more of some of the higher concentrations. Against this possibility is the fact that rats

of this weight are able to excrete far larger amounts of water per day, 180 cc. and more (Richter, '38).

Amount of sugar in grams ingested at the various concentrations

We have seen that the rats drank the largest amounts of sugar solutions at concentrations of 9 to 11%. Does this mean that at this concentration they have reached their capacity for the carbohydrates? Figure 5 gives the average intake

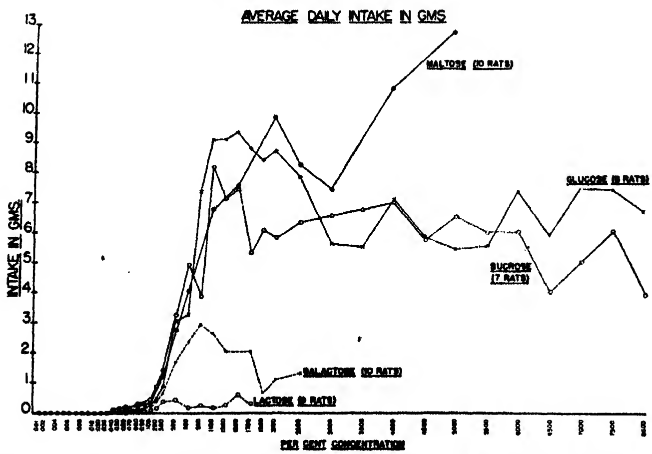

Fig. 5 Curves showing the average daily intake of the five sugars in grams with relation to the concentrations of the solutions. The ordinates give the average daily intake in grams; the abscissae give time in days and concentrations of the solutions in per cent.

in grams of the sugars at the different concentrations. From 2 to 9% the intake of maltose, glucose, and sucrose increased at approximately the same rate. With higher concentrations the intake curves began to diverge. The glucose intake reached a peak of 9.3 gm. at 15%, then decreased until it reached a level near 6 gm. at 30%, at which it remained fairly constant until the concentration reached 80%. Sucrose intake attained a peak of 8.3 gm. at 11% and decreased sharply. From 17 to 60% it remained fairly constant at a level near 6 gm. and then decreased slightly more, finally averaging

about 4 gm. at 80%. In marked contrast, the maltose intake continued to increase at a steady rate up to the saturation concentration of 50%, when it averaged 12.8 gm., over twice as much as the intake of sucrose and glucose at the same concentration. The galactose intake reached a peak of 2.7 gm. at 9% and then decreased gradually until the solution began to crystallize at 25%. The lactose intake attained a peak of 0.4 gm. at 15%.

During all of these tests the rats continued to grow at their normal rate.

DISCUSSION

The results of these experiments have shown that rats have a great appetite for maltose and a lack of appetite for, or an actual aversion to, lactose. It is conceivable that appetite or aversion may in some way depend on the constituents of the basic diet. For instance, a high lactose content may reduce or eliminate the appetite for any additional lactose; or a low maltose intake may stimulate the rats to seek more maltose. However, with its high proportion of graham flour the diet contains approximately 60% carbohydrate chiefly in the form of starch; and with its relatively small proportion of skim milk powder, 10%, or 2% lactose, the diet does not have a superabundance of lactose.

That the rats actually were able to utilize the high amounts of maltose that they ingested we know from the fact that they grew at the normal rate and remained in good health. Whether they would have continued over a longer period of time to take such large amounts of maltose was not determined in this experiment.

On the basis of the observations made over a wide range of self-selection experiments it would seem likely that the great appetite for maltose indicates that the rats are able to utilize maltose better than any of the other sugars tested and that they are least well able to utilize the lactose.

The recent experiments of Maddock, Hawkins and Holmes ('39) indicate that maltose may be utilized readily and directly without being broken down into glucose. These workers showed that in dogs and rabbits hepatectomy

abolished the normal waves of the electro-encephalogram, presumably because in the absence of the liver, some sugars can no longer be metabolized. Injections of maltose, glucose, and mannose into the carotid artery reestablished the normal waves, while lactose and galactose had no effect.

In agreement with the present observations on lactose, we have found in experiments in which rats had access to only one sugar and no other food at all that glucose gave a survival time of 57 days, sucrose 43 days, while lactose and galactose gave only 7.5 and 6.5 days, respectively. It may be that, as Schantz, Elvehjem and Hart ('38) and Schantz and Krewson ('39) have pointed out, lactose cannot be utilized in the absence of certain natural food fats. However, the McCollum diet with its 5% content of butter has an ample amount of natural fats for the utilization of larger amounts of lactose than are contained in the regular food. We must consider also that the appetite for lactose may change with age.

The taste threshold experiments brought out the surprising observation that rats have a lower threshold for maltose than for any of the other sugars. This may mean that they are able to taste it in foods in very small quantities. They recognized maltose solution in concentrations of 1 part of maltose to 2000 parts of water. Apparently, the rats distinguished galactose and lactose from water only when present in much higher concentrations.

It will be of interest to know if these thresholds can be changed by interference with the functions of the pancreas, adrenals, or pituitary. From other self-selection experiments we know that nearly all pancreatectomized rats have an aversion to sucrose. It is possible that they may be attracted to one of the other sugars.

The fact that the rats showed the maximum preference for most of the sugars at concentrations of 8 to 11% is of interest since, according to Engel ('28) and others, most drinks—coffee, tea, etc.—used by humans have an average concentration of 9% sucrose. Krogh-Jensen, working with Pauli ('25), found experimentally by actual measurements that

humans prefer sucrose solution at 9%. Engel found that at 9% all of his subjects stated that they liked sucrose solution, while with lower and higher concentrations some individuals found the taste unpleasant.

In preliminary experiments made on humans we have found that in the higher concentrations, approximately 50%, humans also had a definite preference for maltose. At 50% they actually stated that the maltose had a delicious flavor. This holds only for maltose that has been freed from the characteristic flavor of malt which gives less purified preparations such a penetrating taste. We used only the purified preparations.

SUMMARY

1. Rats distinguished between sugar solution and distilled water in the following concentrations:

	%
Maltose	0.06
Glucose	0.20
Sucrose	0.57
Galactose ...	1.60
Lactose	——

2. They showed the greatest preference for maltose, next for glucose and sucrose, only a slight appetite for galactose, and none for lactose.

3. The animals exhibited the greatest preference for solutions of the various sugars when offered in concentrations near 10%. This agreed closely with concentrations of sugar solutions most commonly used by humans to sweeten drinks.

4. On the basis of results of previous self-selection reports it was concluded that, when offered in purified form to adult animals, maltose may be utilized better than the other sugars, lactose least well.

We wish to thank Dr. C. S. Hudson of the National Institute of Health and Dr. Katherine Rice of the Johns Hopkins Medical School for their helpful suggestions and criticisms. We also wish to thank Mr. B. S. Buckmaster of the Pfanstiehl Chemical Company for making it possible for us to purchase some of the sugars.

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THE PLANE OF INTAKE OF BEEF MUSCLE PROTEIN AS AFFECTING THE ENERGY AND THE NITROGEN METABOLISM OF THE MATURE ALBINO RAT[1]

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The influence of the plane of intake of protein in the form of casein, and as present in beef muscle, as affecting the utilization of food energy and protein by the growing albino rat having been investigated at considerable length in this laboratory (Forbes, Swift, Black and Kahlenberg, '35; Forbes, Voris, Bratzler and Wainio, '37; Forbes, Black, Thacker and Swift, '39), the purpose of the study to be discussed was to investigate the same matter with adult rats as the experimental subjects in order to learn how the plane of intake of protein affects animals of such maturity that they are no longer able to store protein in significant quantities.

Among the possible applications of the results of this series of studies, one that has been prominent in the minds of the experimenters is their bearing on the protein content of diets for human beings—growing and adult—especially at environmental temperatures above the zone of thermal neutrality; the present unit of this series, therefore, is significant in relation to mature human beings.

With the approach of summer weather, writers of newspaper health columns frequently advise restriction of the content, in the diet, of protein in general, but of meat in particular, with the idea of diminishing the heat production, and thus avoiding

[1] Authorized for publication on February 7, 1940, as paper no. 955 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

discomfort and possible prostration as a result of the overtaxing of the powers of adaptation to elevated temperatures. A question of importance, from several points of view, is whether this advice is founded on correct or on incorrect understanding.

The three published papers referred to cover, in all, five experiments. In the first and second of these studies the diets contained 10, 15, 20 and 25% of protein, respectively, this protein being almost exclusively casein; in the third experiment, also with casein, the diets contained 25, 30, 35 and 45% of protein, respectively; and in the fourth and fifth experiments the protein was mainly that of beef muscle, the diets containing 10, 25, 35 and 45% of protein, respectively.

Among the findings from these experiments with growing rats were the following: Corresponding to progressively greater protein contents of equicaloric diets, from 10% to 45%, were increased digestibility and decreased metabolizability of food energy; decrease in heat production at a diminishing rate of decrease; increase in energy of urine; increase in gain in body weight and in energy of body gain until the optimum proportion of protein in the diet was reached, and, with further increase in protein, slight decreases in rate of gain in weight, energy, nitrogen and fat, and in fat gained per gram of nitrogen gained. Also, it was shown that in the assumedly complete diets used, with growing rats as subjects, beef muscle protein does not have a greater heat stimulating effect than does casein.

The most striking observation was that in these equicaloric diets, differing as to protein content, there were not in evidence dynamic effects corresponding to the differences in protein intake.

Apparently the difference in heat production from a high-protein diet and a low-protein diet of the same gross energy value is the resultant of several factors—difference in the metabolizable energy of the diets; difference in the synthesis of food nutrients into body substance, thus leaving different amounts of nutriment available for heat production; and pos-

sible differences in the basal metabolism, and in heat increments due to the food and to the voluntary activity.

It should be realized that the difference in heat increment accompanying the difference in protein intake is not an effect of an addition of protein to the low-protein diet, but is the result of the substitution of protein for equicaloric carbohydrate and fat; and also that this difference in heat production is not a difference in the sums of the separate dynamic effects of the associated nutrients, but in the dynamic effects of the diets as entities.

With this understanding, derived from experiments on growing rats, the present experiment with mature rats was undertaken.

The principle of the body balance method, by which this investigation was conducted, is that food energy minus energy of excreta and of body gain equals heat production.

This procedure was first employed by Swift, Kahlenberg, Voris and Forbes ('34). While the measurement of heat production is indirect, the determinations on which it depends are highly consistent, and the single value for the heat produced in an experimental unit during a long period of observation possesses an accuracy and a significance as representing the total of vital activities unquestionably greater than would characterize any short-time measurement raised, by multiplication by a large number, to a value representing an equally long period.

The rats used as subjects in this investigation were chosen from individuals aged 255 to 273 days, the extremes in live weight being 337 and 410 gm. Ten quadruplets of male rats, four from each of ten litters, were selected, three from each quadruplet to be fed, and one to serve as a member of the control group which was killed and analyzed at the beginning of the experiment to give data on the initial status of those which were fed, thus providing a basis for the determination of body gains during the experiment. Actually, however, the control group contained only nine rats, the tenth having been sacrificed by error.

The experimental diets employed were similar in nature to those used in the previously published units of the general program of studies. Their composition is given in table 1.

The objective being equicaloric diets containing 10, 25 and 45% of protein, respectively, these mixtures were based on constant proportions of "Cellu flour," Osborne and Mendel salt mixture, sodium chloride, butterfat and yeast, and different proportions of dextrin, "Crisco" and dried beef muscle, the resulting diets containing, by analysis, 10.13%, 25.06% and 44.88% of protein, respectively, and 4785 calories of gross energy per gram.

TABLE 1

Make-up of diets

	DIET 1	DIET 2	DIET 3
	%	%	%
Cellu flour	4.00	4.00	4.00
Salt mixture [1]	4.00	4.00	4.00
NaCl	1.00	1.00	1.00
Butterfat	1.00	1.00	1.00
Yeast [2]	6.00	6.00	6.00
Dextrin	60.18	47.35	30.23
Beef muscle	8.58	27.94	53.77
Crisco	15.24	8.71	0.00
Total	100.00	100.00	100.00

[1] Osborne and Mendel.

[2] The yeast used was a mixture of 5 parts brewer's yeast and 1 part irradiated yeast; and carotene, in oil, was added to each diet in a quantity providing 1.4 U.S.P. units of vitamin A equivalent per gram.

All rats were given 1 week's preliminary feeding before beginning the experiment proper, with ten rats on each of the three diets, and with the controls on a mixture of equal parts of each of the three diets.

During the 10 weeks' experimental feeding the subjects were fed once a day, with triplet food control, the quantity given being determined, from day to day, by the amount of the ad libitum food consumption of the rats on the diet consumed in the smallest quantity—this being nearly always the 10% protein diet.

The average daily food consumption for the experiment as a whole was approximately 13 gm., which proved to be only slightly more than the maintenance requirement, since the average initial and final live weights were 388 and 393 gm., respectively.

At the end of the experiment the rats were killed by anaesthesia, and the bodies were comminuted, dried in vacuum, extracted with ether, ground, and analyzed in the usual manner, the entire bodies being accounted for in two samples—ether extract, and extraction residue.

The reduction of the dried and extracted bodies to a satisfactory condition for sampling and chemical analysis was accomplished by grinding several times through an "Excelsior" laboratory mill. In order to serve this exacting purpose, however, it is necessary to babbitt the interior of this mill in such manner as to fill certain spaces in which material to be ground would otherwise lodge; also the efficiency of the mill was increased by fastening a small wing of sheet metal onto the rotating shaft in such position that it agitated the material to be ground; and further, the cutting up of the dried skin, with shears, was necessary in order to prepare it for passing between the burrs of the mill. The shearing action of the interlocking teeth of the burrs of this mill serves to reduce the dried and extracted flesh, skin, hair, bones and teeth, all together, to a perfect condition for sampling and chemical analysis.

At the time of sacrificing the experimental subjects, gross examination revealed a pathological condition of the lungs of several of the rats. The specific character of this disorder was not determined, since the method of experimentation required that all parts of the bodies be accounted for by chemical analysis. The observed abnormalities are believed to have been caused by colds; however, no evidence was obtained to show that this condition appreciably affected the conclusions drawn from the results of this study.

The averages of the analytical data from the control group were as follows: live weight 373 $\pm$ 11 gm.; empty live weight

362 ± 11 gm.; ether extract 31.57 ± 2.74 gm.; air-dry extraction residue 106.2 ± 2.92 gm.; total nitrogen 12.8 ± 3.9 gm.; and total energy 751 ± 35 Calories.

In all tables the ± values signify probable errors.

The quantities of food eaten, the initial and final live weights, and the gains in empty body weight, in nitrogen and in fat are presented in table 2.

In computing these gains it was assumed that the initial composition of each of the subjects of experimentation was the same as the average composition of the nine individuals of the control group.

Since the experimental subjects were mature, but were fed virtually as much as they would eat, it is natural that they

TABLE 2

Food eaten, and gains in live weight, nitrogen and fat during 70 days

PER CENT OF PROTEIN IN DIET	FOOD EATEN, DRY MATTER	INITIAL LIVE WEIGHT	FINAL LIVE WEIGHT	GAIN IN EMPTY BODY WEIGHT	NITROGEN OF BODY GAIN	FAT GAINED
	gm.	gm.	gm.	gm.	gm.	gm.
10.13	837	385 ± 4	391 ± 7	8 ± 3	− 0.2 ± 0.1	11.53 ± 1.72
25.06	835	388 ± 3	401 ± 6	15 ± 6	0.1 ± 0.2	12.06 ± 3.76
44.88	837	390 ± 3	388 ± 7	− 2 ± 6	0.1 ± 0.2	− 0.18 ± 2.19

were essentially in nitrogen equilibrium. The rats on the 10% and the 25% protein diets, however, were able to gain small quantities of fat, but there was no gain of fat from the 45% protein diet, because of its lower metabolizability.

In table 3 is represented the distribution of food energy as affected by the plane of protein intake.

The positive balance of protein is shown to have been insignificantly small from all three diets, and a slight body gain in energy from the two lower-protein diets is shown to have been in the form of fat.

The progressive replacement of dextrin and Crisco in the 10% protein diet with dried beef muscle, for the production of equicaloric diets containing from 10% to 45% of protein, resulted in increases in the digestible energy of the diets

from 3722 Calories to 3808 Calories, as totals per rat for the 70-day experimental period; but the coincident increase in urinary energy more than offset this increase in digestible energy, with the result that in the order of the increase in dietary protein from 10% to 45%, there was a decrease in the metabolizable energy of the diets from 3619 Calories to 3407 Calories; and accompanying this decrease in metabolizable energy was a decrease in heat production from 3505 Calories to 3402 Calories, for the entire experimental period.

The odds that the heat from the 45% protein diet was less than from the 10% protein diet were 147 to 1. The odds that

TABLE 3

Distribution of total food energy as affected by the plane of protein intake

PER CENT OF PROTEIN IN DIET	10.13	25.06	44.88
Energy value in calories:			
Food	4220	4220	4220
Feces	498 ± 8 [1]	403 ± 5	413 ± 6
Digested material	3722 ± 48	3817 ± 50	3808 ± 50
Urine	103 ± 1	237 ± 3	401 ± 4
Metabolized material	3619 ± 47	3579 ± 48	3407 ± 46
Body gain: total	113 ± 18	124 ± 36	5 ± 25
as protein	9 ± 4	14 ± 6	12 ± 8
as fat	104 ± 16	110 ± 35	− 6 ± 21
Heat production	3505 ± 36	3456 ± 26	3402 ± 43

[1] All ± values signify probable errors.

the 25% protein diet produced less heat than the 10% protein diet, and that the 45% protein diet produced less heat than the 25% protein diet, however, were only 5 to 1, and 4 to 1, respectively, and, therefore, were not significant. These odds were computed by "Student's" method.

While this decrease in heat production is conceivably the resultant of several contributing factors, prominent among these is the coincident decrease in the metabolizable energy of the diets, in harmony with the fact that whereas protein has a gross energy value of about 5.7 Calories per gram, it has a value of only about 4.2 Calories per gram to produce heat in

the body, the metabolizable energy of carbohydrate and fat being more nearly as great as their gross energy values.

The percentages of the metabolizable energy that were expressed as heat were 96.8 ± 0.5, 96.6 ± 1.0 and 99.9 ± 0.8, respectively, in the order of the increasing protein contents of the diets. Thus, though there was a slight decrease in heat production in the order of the increase of dietary protein, these values constituted a slight increase in heat as a percentage of the metabolizable energy.

This finding is not necessarily out of harmony with the measurements of the specific dynamic effects of nutrients which have been published from this laboratory, since the experimental backgrounds in these two heat measurements have differed so radically that the results are not of the same significance.

Among a number of papers reporting specific dynamic effects of protein, a recent one by Forbes, Bratzler, Thacker and Marcy ('39) is of interest. In this study the dynamic effect of protein was determined by superimposing it upon a basal, maintenance diet, in 6-hour periods of measurement in the absence of voluntary activity; while in the present study the protein was introduced into the diet not by superimposition but by substitution for an equicaloric quantity of nonnitrogenous nutriment—this last having a higher metabolizable energy value than the protein introduced—the heat measurement representing 70 days' feeding under normal conditions of activity. It would be impossible to say what the consequences of this difference in conditions of experimentation should be in relation to the differences in heat production ascribable to the protein.

The lack of complete agreement between the very small values for grams of gain of protein and of fat, as given in table 2, and the similarly small values for calories of gain as protein and as fat, as given in table 3, results from the fact that these two sets of values were determined independently, those in table 2 as results of Kjeldahl determinations of nitrogen and of ether extract determinations of fat, and those in

table 3 as results of bomb calorimeter determinations of the energy of the ether extract and of the extraction residue, which is designated protein.

Referring to table 4, the apparent digestibility of the food nitrogen varied in the same order as the intake, but not in the same proportion, the digestibility of the protein of the 10% protein ration being 83.1%, and of the 45% protein ration 92.9%—this difference presumably signifying, at least in part, increased intake in excess of the amount eliminated as products of endogenous metabolism.

TABLE 4

Distribution of food nitrogen as affected by the plane of protein intake

PROTEIN IN THE DIET IN PER CENT	10.13		25.06		44.88	
Nitrogen:						
Food in grams	14.3		35.4		63.3	
Feces in grams	2.4	± 0.0 [1]	3.3	± 0.1	4.5	± 0.1
Digested: In grams	11.9		32.1		58.8	
In per cent of food N	83.2	± 0.2	90.7	± 0.1	92.9	± 0.1
Urinary: In grams	10.7		30.6		57.6	
In per cent of food N	74.8	± 0.8	86.4	± 0.3	91.0	± 0.4
Gain of nitrogen	– 0.2	± 0.1	0.1	± 0.2	0.1	± 0.2
Urinary nitrogen per calorie of urinary energy	0.104	± 0.001	0.129	± 0.001	0.144	± 0.001

[1] All ± values signify probable errors.

The fecal outgo of nitrogen was remarkably low, and the urinary nitrogen very high, the balance being approximate equilibrium.

The material increase in urinary nitrogen per calorie of urinary energy accompanying the increase in dietary protein from 10% to 45% is understood to signify an increasing proportion of the nitrogenous outgo in the form of urea.

The percentages of the heat production derived from the oxidation of protein were nearly the same as the percentages of protein in the three diets, namely, 8.1, 23.5, and 44.9.

In relation to the accounting for the food nitrogen in the products derived therefrom, the amounts by which the nitrogen intake per rat, during the 70-day experiment, exceeded the

nitrogen of the feces, the urine and the body gain were 1.41, 1.31, and 1.19 gm., for the 10%, the 25% and the 45% protein diets, respectively, these quantities being equivalent to 10%, 4% and 2%, respectively, of the food nitrogen.

These nitrogen losses did not represent scattered food, because food was not scattered in significant quantities, and because the relative quantities of nitrogen lost were not in accord with the nitrogen contents of the diets. Neither were these losses of nitrogen in the form of urinary ammonia, because the quantities lost differed greatly in nitrogen content, and also because the quantities of these losses of nitrogen differed in the reverse order from that of the nitrogen contents of the diets. Moreover, these losses could not have been in feces, because they were collected with accuracy; nor is it believed that they represent error in the determination of the body gain of nitrogen—since a body gain in this constituent was neither expected nor found.

By exclusion, therefore, it is concluded that these losses of nitrogen represented shed hair and scurf, which were present in considerable quantities with these mature rats, but were not collected and analyzed.

The differences between the amounts of nitrogen not accounted for, however, would not represent significant amounts of energy, in relation to the total heat production, if these losses, as assumed, represented hair and scurf, because the ratio of energy to nitrogen in hair is materially less than the equivalent value representing the diets.

By way of recapitulation of the six experiments of which the present one is the last, in the first five the subjects were growing, and in the last, mature; in the first three experiments the principal protein of the diets was casein, and in the last three, beef muscle; the range of protein contents of the diets in the first two experiments was 10% to 25%, in the third, 25% to 45%, and in the fourth, fifth and sixth, 10% to 45%, though in each experiment all diets had the same gross energy content; in all experiments there was decrease in the heat production of the subjects in the order of the increasing protein contents of

the diets, though these factors were not closely proportional the one to the other; the body gain of energy increased with increasing protein contents of the diets until the optimum was reached, and then decreased in the order of further increase in the protein contents of the diets; determinations of final basal metabolism were made in some of the experiments, but the systematic factoring of the heat production into basal metabolism, heat increment due to the diet, and heat increment due to voluntary activity was impracticable in connection with the experimental procedure employed and was not attempted.

In relation to the cause of the decrease in heat production in the order of the increasing protein contents of the diets—the metabolizable energy obviously dominates this function in the sense that no energy can be expressed as heat which is not metabolizable, but the utilization of metabolizable energy for body gain prevents its expression as heat, and in four of the experiments among the six the decrease in heat production in the order of increasing protein contents of the diets exceeded the accompanying decrease in metabolizable energy. In these four instances among the six, therefore, the metabolizable energy did not exert a clear dominance over the heat production.

In the light of the results of this investigation as a whole, it is suggested that in relation either to growing or to mature human beings it would be ineffective to diminish the proportion of protein to nonprotein material in the diet as a means of minimizing heat production during excessively warm weather; and that the logical procedures for the accomplishment of this purpose would be to avoid unnecessary activity, to be conservative as to the total quantity of food eaten, and to keep the caloric value of the food relatively low by increasing the proportions, in the diet, of watery foods such as fruits and vegetables.

In regard to fat in the hot-weather diet, conservatism in the intake of fat is desirable because of its high caloric value, but the usual fat content of the diet should not be decreased without consideration of the amount of vitamin A that would remain.

SUMMARY

The effects of the plane of protein intake on the utilization of the food protein and energy of equicaloric diets containing 10%, 25% and 45% of protein, respectively, were investigated in a 70-day balance experiment with mature albino rats as subjects.

The rats remained in approximate equilibrium with respect to live weight and to the nitrogen content of their bodies.

The digestibility of the protein of the diets increased in the order of the increase in dietary protein; and the digested nitrogen and the urinary nitrogen were essentially the same values.

The metabolizable energy diminished in the order of the increasing protein contents of the diets, primarily because the increase in the outgo of urinary energy more than counterbalanced the coincident decrease in feces energy, from the two higher protein as compared with the lowest protein diet.

The heat production diminished, slightly, in the order of the increase in protein in the diets, this diminishing heat production being equivalent to a slightly increasing heat in relation to the metabolizable energy.

Urinary nitrogen per calorie of urinary energy increased in the same order as the increase in dietary protein.

The bearing of the results in relation to human diet is pointed out.

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HYPERALIMENTATION IN NORMAL ANIMALS PRODUCED BY PROTAMINE INSULIN [1]

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THREE FIGURES

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Early in its history insulin was used in an attempt to improve the nutrition of non-diabetic patients (Pitfield, '23; Marriott, '24). It was regarded as stimulating the appetite thus leading to a higher caloric intake of food. It has been used for this purpose by many although proof of its efficacy was lacking. The reason for this is because of the many factors involved in such clinical observations, but the careful and well controlled observations of Freyberg ('35) are particularly interesting. His investigation failed to support the prevalent opinion that insulin has any significant influence upon the appetite. That ordinary insulin has no influence on body weight of rabbits has also been reported as the conclusion from experiments by Long and Bischoff ('30). The rat more closely resembles man in its dietary habits and the nature of its gastro-intestinal tract than does the rabbit. Because of the favorable reports in man it appeared desirable to repeat this study in the rat.

REGULAR INSULIN [2]

The first experiments were carried out in 1929 with the regular commercial insulin then available in a strength of 40

[1] Preliminary reports of the experiments described here were given before the Society for Experimental Biology and Medicine (Proc. Soc. Exp. Biol. and Med., vol. 36, p. 406, 1937) and the meeting of the American Association for the Advancement of Science in Denver, June, 1937.

[2] The regular insulin supplied to us in 1930 and the protamine zinc insulin used in the first part of the experiments with this product were furnished by Eli Lilly and Company of Indianapolis to whom we are indebted. Additional supplies with which to complete the work were provided by E. R. Squibb and Sons.

units per cubic centimeter. Either 3 units or 10 units were given in the morning. Results in three individual rats are charted in figure 1. They are typical of all of our data obtained with regular insulin. The diet was supplied ad libitum and was composed of powdered commercial casein 25, starch 40, lard 10, butterfat 15, tested dried brewers' yeast 5 and Osborne and Mendel's standard salt mixture 5. There is no indication that either the food intake or the body weight was augmented as a result of the administration of regular insulin. This confirms the conclusions that have been reached in studies with

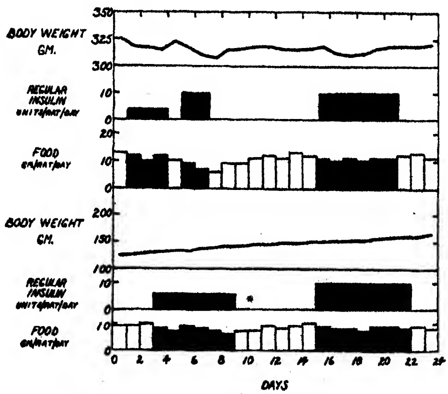

Fig. 1 Experiments illustrating the failure of regular insulin to influence the food intake or body weight of the rat. The first experiment is from data obtained on a single rat while the second represents the averages from a group of three.

the rabbit (Long and Bischoff, '30). In contrast to these results Macleod et al. ('30) concluded from experiments on pigs that "insulin had only a very slight accelerating effect on the rate of increase in weight . . . ." but an examination of their data gives no support whatever to even this questionable positive conclusion. Maclagan ('37) has noted a slight augmentation of the appetite of rabbits with insulin which was presumably of the ordinary type but this was only over a period of a few hours without any evidence that it could be continued from day to day by insulin administration.

PROTAMINE ZINC INSULIN

This slowly absorbed insulin preparation was given subcutaneously in a strength of 40 units per cubic centimeter. In every case the use of this insulin in substantial doses led to an increased appetite (fig. 2). In some cases the increase in food intake was tremendous (table 1). The excess food was largely stored in the form of fat. The relation of the weight increase

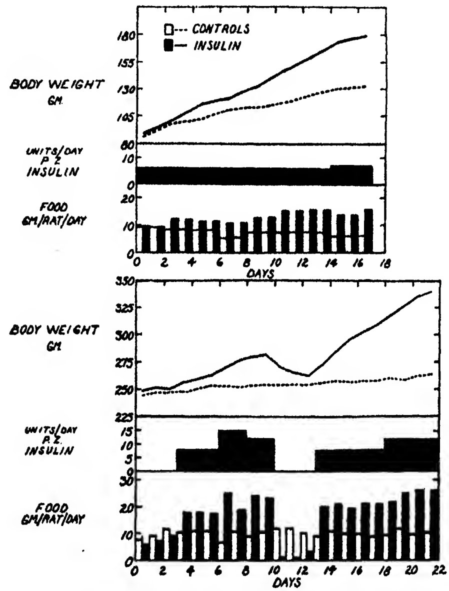

Fig. 2 Typical examples of the marked effect of protamine zinc insulin on the food intake and body weight of the rat. In both experiments the figures are averages for groups of four rats.

to the excess food consumption of comparable control rats shows that no considerable proportion of the excess calories is dissipated in an increased metabolism. There would naturally be the higher specific dynamic action of more food. We have no data on the metabolic rate. One point of interest was the 5 to 20% increase in the weight of the intestinal tract depending on the extent of the increased food intake.

APPETITE AND BLOOD SUGAR

Hypoglycemia or a more moderate reduction in the blood sugar level appears to be the chief factor in the weight gain due to protamine insulin. Frequent blood sugar determinations are not feasible in the rat but the data in figure 3 show a very definite inverse relation between the blood sugar level and the food intake in the rabbit. The food consisted of ordinary alfalfa hay and oat commercial rabbit pellets. It is true,

TABLE 1

The increase in food intake and body weight after protamine insulin

EXP. NO.	NUMBER OF RATS IN EACH GROUP	SEX	PERIOD IN DAYS	CONTROL AVERAGES: Initial body weight	Final body weight	Increase in body weight per day	Food intake per day	PROTAMINE INSULIN TREATED AVERAGES: Protamine insulin per rat per day	Initial body weight	Final body weight	Increase in body weight per day	Food intake per day	INCREASE PER DAY DUE TO PROTAMINE INSULIN: Food intake	Body weight
				gm.	gm.	gm.	gm.	units[1]	gm.	gm.	gm.	gm.	gm.	gm.
1	4	♂	8	57	75	2.25	6.1	2	60	80	2.50	8.0	1.9	0.25
2	5	♂	11	239	243	0.40	12.0	4	242	274	2.00	19.0	7.0	1.60
3	6	♂	20	240	261	1.05	11.2	10	242	308	3.30	16.8	5.6	2.25
4	3	♂	15	94	161	4.45	9.9	6	97	202	7.00	13.8	3.9	2.55
5	4	♀	15	86	132	3.07	7.6	6	89	178	6.00	12.6	5.0	2.93
6	4	♂	4	98	113	3.75	9.5	8	99	126	6.75	13.5	4.0	3.00
7	4	♀	4	88	101	3.25	8.1	8	92	118	6.50	13.1	5.0	3.25
8	6	♀	5	159	163	0.80	8.0	8	162	183	4.20	12.5	4.5	3.40
9	4	♀	3	204	206	0.66	9.3	12	202	227	8.33	10.1	10.8	7.67

[1] The protamine insulin dose was always divided, half of it being given at either end of the day.

of course, that the concentration of insulin in the organism is probably the highest when the blood sugar is the lowest and that insulin concentration might be the factor which governs the appetite of these animals. Other observations make this seem unlikely. It might be mentioned that the rabbit is not a very good animal for the type of experiment in which we are interested here and can never be made to gain much weight with insulin administration. The reason for this is the low energy value of its food when calories per unit volume are

considered. Rats given a very bulky diet gain slightly at first and then die in hypoglycemia because they are unable to eat enough.

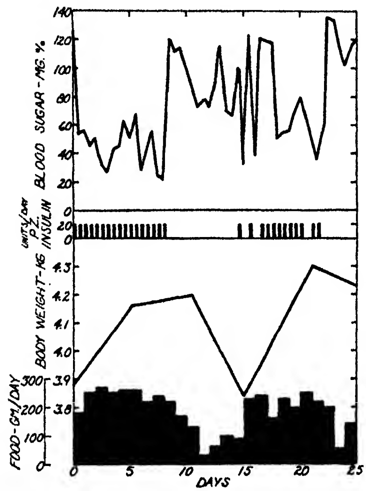

Fig. 3 The influence of protamine insulin injections upon the blood sugar level, food intake and body weight in a female rabbit. This demonstrates the typical inverse relation between the blood sugar level and the food intake.

PROTAMINE INSULIN ACTION AND ADRENAL MEDULLA

The hypoglycemia produced by insulin induces an increased secretion of epinephrine (Cannon, McIver and Bliss, '24). This mobilizes sugar from the liver and aids in counteracting the hypoglycemia. The increase in the blood sugar level which normal rats show in response to emotional excitation does not occur if the adrenal medullas have been destroyed by enucleation (Harris and Ingle, '37). If the lowered blood sugar level rather than the increased insulin concentration is the factor governing the increased appetite after protamine zinc insulin, the weight gain should be greater when insulin is given after enucleation of the adrenal medullas. It can be seen from table 2 that protamine insulin is more effective under such circumstances. The medullas were enucleated and a month allowed for the cortical tissue to regenerate before this experiment

was carried out. The insulin treated rats without their adrenal medullas had lower blood sugars than the others and had a greater tendency to die in hypoglycemia than control rats receiving the same amount of insulin.

TABLE 2

Influence of removing the adrenal medullas on the weight increase due to protamine insulin. The dose of insulin was the same for every rat. They were given 5 units twice daily for a period of 20 days. Each figure is an average for a group of six rats

GROUP	INITIAL BODY WEIGHT	FINAL BODY WEIGHT	WEIGHT INCREASE	
	gm.	*gm.*	*gm.*	%
Male controls	253	330	77	30
Males with medullas removed	257	380	123	48
Female controls	197	265	68	35
Females with medullas removed	194	284	90	46

DISCUSSION

We cannot be absolutely certain at the present time whether the increased appetite which results from protamine insulin administration is due to the reduction in the blood sugar level or the concentration of insulin circulating in the organism, although we favor the former possibility. Assuming the decrease in the blood sugar level to be the cause, there is the question of whether its action is peripheral, through an increase in gastric hunger contractions, or central by its influence on a possible appetite center in the brain. The work of Bulatao and Carlson ('24), who found that gastric motility can be increased by depressing the blood sugar with insulin and abolished by the administration of glucose, would point to direct action on the stomach but Mulinos ('33) could find no relation between gastric motility and normal spontaneous variations in the blood sugar. It is likely that the variations under ordinary conditions would be too small to give a satisfactory correlation.

We indicated in our introduction that although ordinary insulin has never been proved to have any influence on the appetite it has often been used to improve the food intake in

man. This subject is well reviewed by Marlow and Callaway ('38). They found that unlike ordinary insulin the slow acting protamine zinc insulin was really efficacious in increasing the appetite and causing a gain in weight. The action of ordinary insulin on the blood sugar is apparently too transient in the fed organism to influence the blood sugar level for an appreciable length of time. The slowly absorbed protamine compound is much more efficacious in the rat than in man insofar as augmenting the food intake is concerned. Emotional inhibition of gastric motility and hence appetite is undoubtedly of greater importance in the case of man than the rat.

The doses of insulin which were used are not very great when we consider the pharmacology of this agent in the rat, but transferred to other species on a body size basis these doses might be enormous. This is true in the case of humans where an infinitely smaller dose of protamine zinc insulin is necessary to reduce the blood sugar level enough to influence the food intake (Marlow and Callaway, '38). We desire to point this out because of the well known dangers of hypoglycemia. Even though there may be apparent recovery from hypoglycemic symptoms a reduction in the blood sugar level for any extended period of time is very apt to lead to permanent damage to the brain (Sherrill and MacKay, '39).

SUMMARY

Regular insulin in doses of 1 to 5 units per 100 gm. body weight per day had no measurable effect upon the food intake or body weight of albino rats. The slowly absorbed protamine zinc insulin in similar doses had a remarkable influence upon the appetite. The food intake could be more than doubled in this manner leading to large increases in body weight.

The chief factor leading to the increased appetite appeared to be the decrease in the blood sugar level, for an inverse relationship between the latter and the food intake was demonstrated in the rabbit. Furthermore removal of the adrenal medullas, thus preventing the secretion of epinephrine when stimulated by hypoglycemia, and the ensuing blood sugar-

raising effect led to greater weight gains from a given dose of insulin.

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INTESTINAL ABSORPTION OF VITAMIN A IN THE NORMAL RAT [1]

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Evidence is accumulating which indicates that a higher conversion factor must be used for converting the extinction coefficient, $E^{1\%}_{1\,cm.}$ at 328 millimicrons, of esters of vitamin A to biological (International or U.S.P.) units than for vitamin A alcohol (Morgan, Edisbury and Morton, '35; Barthen and Leonard, '37; Moll and Reid, '39; Koehn and Sherman, '40). Attempts to establish a sound physiological basis for this difference have not been strikingly successful. It occurred to us that as a first step toward an understanding of this phenomenon, information as to the mechanism of vitamin A absorption from the intestine was needed. The study reported herewith was designed, primarily, to determine whether vitamin A esters, like other esters of the fatty acids, are hydrolyzed by the intestinal enzymes prior to absorption as well as to indicate the state of the vitamin in the gut wall during absorption.

EXPERIMENTAL

The general plan of the experiment consisted in setting up comparable groups of rats which were killed at stated intervals after the administration of a known dose of vitamin A ester. The residual intestinal contents, as well as the gut walls, were analyzed for total vitamin A and for percentage of vitamin

[1] This paper was read before the thirty-fourth annual meeting of the American Society of Biological Chemists, Inc., at New Orleans, March 15, 1940.

A alcohol. The extracts from the wall were also analyzed for free fatty acid.

PROCEDURE

Thirty-six young, adult, female rats (Wistar strain) were divided into nine groups of four each. The animals were 90 days old, averaged 145 gm. in weight and were 16 hours post-absorptive at the time of the experiment. Each rat was given a dose of 54,160 U.S.P. units of vitamin A as distilled ester concentrate from fish liver oil dissolved in 1.0 cc. of corn oil [2] by stomach tube. The free alcohol content of the preparation was less than 1%. At stated intervals after the injection of the dose, the animals in each group were etherized and the abdominal cavity exposed by a mid-line incision from the costal margin to the perineum. The gastro-intestinal tract, from the esophagus to the ileo-cecal junction, was securely ligated and removed intact. The large intestine was not taken. As much of the mesenteric fat as possible was stripped from the intestine although the removal was not absolutely complete. The contents of the stomach and intestine were washed out with several successive 5 cc. portions of normal saline which was introduced into the stomach with a syringe and carefully propelled through the length of the gut by stripping between the fingers. Gut contents and washings from the four rats in each group were pooled and extracted three times with ethyl ether in separatory funnels. The ether extracts were dried over anhydrous sodium sulfate and the total lipid analyzed after the removal of the solvent under a stream of nitrogen on the steam bath. The results are summarized in the first part of table 1. The per cent of vitamin A alcohol was determined by the technique of analytical distillation (Hickman, '36, '37). The distillate fractions were taken at 10° intervals from 90 to 240°C. and at pressures ranging from 4.0 to 1.9 μ (Parani gauge attached to still chamber). Under the conditions of distillation employed, vitamin A alcohol comes over below 150° while the

[2] The commercial corn oil, Mazola, was used.

peak of the ester elimination curve occurs between 200 and 210°. The alcohol: ester ratio of the sample is easily calculated from the areas under their respective maxima of the elimination curve.

The pooled gastro-intestinal tissue from each group was minced with scissors, ground with sand, transferred quantitatively to Soxhlet thimbles and extracted in hot-vapor percolators for 8 hours with 95% alcohol and then for a similar

TABLE 1

Recovery of vitamin A from gastrointestinal contents and tissues after administration of 216,600 U.S.P. units (total) to groups of four post-absorptive rats

GROUP	TIME AFTER DOSING	TOTAL LIPID	TOTAL VITAMIN RECOVERED: Per gram of lipid	TOTAL VITAMIN RECOVERED: Of total fed	VITAMIN DISTILLED [1]	RECOVERY IN DISTILLATES: Total	RECOVERY IN DISTILLATES: As vitamin A alcohol
			From intestinal contents				
	minutes	*gm.*	*units*	*%*	*units*	*%*	*%*
1	80	1.95	44,500	40.1	84,700	110.5	4.36
2	119	1.94	49,200	44.1	93,100	88.3	7.26
3	160	1.57	58,200	42.3	87,800	87.2	8.26
4	191	2.05	57,350	54.4	112,300	86.7	14.10
5	220	1.91	60,000	53.1	110,000	95.5	12.16
6	248	1.44	62,250	41.5	87,100	91.7	10.42
7	277	1.06	77,900	39.3	82,100	80.1	14.10
8	337	1.32	63,100	38.6	79,300	112.7	16.28
9	398	0.87	75,200	30.3	62,700	101.5	16.20
			From G.I. tissue				
1	80	1.97	3650				
2	119	1.48	1750				
3	160	0.95	3420		22,400	88.6	59.20
4	191	1.42	4140		(free fatty acids = 39.2%)		
5	220	1.59	3590				
6	248	1.17	4650				
7	277	1.72	4520		28,100	96.4	81.90
8	337	2.39	3250		(free fatty acids = 48.20%)		
9	398	1.81	5140				

[1] Prior to distillation, all samples were made up in a definite amount of constant yield diluent oil. The values in this column represent the total vitamin A actually placed in the still as determined by direct analysis of the diluted sample. Since a small but accurately known loss occurs on transfer of the sample to the still reservoir, these values are slightly less than the total vitamin recovered as indicated by the product of the figures in columns 3 and 4.

period with ethyl ether. The combined alcohol-ether extracts were evaporated under nitrogen on the steam bath to a watery residue which was transferred to separatory funnels and reextracted with petroleum ether (redistilled, b.p. 38–45°). The solvent was again removed under nitrogen and the total lipid weighed and analyzed for total vitamin A. The results are given in the second part of table 1. Due to the small amounts of vitamin found in the individual group extracts, it was necessary to combine groups 1 to 5 and 6 to 9 in order to carry out the analytical distillation for the separation of the ester and alcohol. Thus, only two composite values for vitamin A alcohol of the G.I. tissues were obtained corresponding to the first and second halves of the experimental period, respectively. They are, however, of sufficient significance to justify including them. Free fatty acid content of the total lipid of the two composite samples was determined by titration with alcoholic NaOH. The values are indicated in the table.

It should be added here that the total vitamin A values for the lipid of the gut contents were determined by ultra-violet absorption ($E^{1\%}_{1\,cm.}$ at 328 millimicrons) using the Hilger medium quartz spectrograph (E-498) in conjunction with the Spekker photometer. The values for the tissue lipids and for the distillate fractions were obtained by means of the antimony trichloride reaction using the Evelyn photo-electric colorimeter (Dann and Evelyn, '38). It has been our experience that the two methods are consistent to about ±15%.

DISCUSSION

Data furnished by studies of this type are of interest with respect to the mechanism of vitamin A absorption from the gut. The difference between biological conversion factors for ester and alcohol has been mentioned. These data indicate that vitamin A esters behave as do other esters of the fatty acids in the intestinal tract and show that they are certainly capable of hydrolysis by the enzymes of the intestinal juice. The additional demonstration that during the height of ab-

sorption, the vitamin within the gut wall exists mainly in the unesterified form can be taken as a potent argument against any assumption that the esters, as such, are directly absorbed from the lumen into the blood. The fact that vitamin A has been largely reesterified by the time it appears in the circulating blood (Clausen et al., '40), however, is quite consistent with generally accepted views of fat metabolism.

Lovern and his associates ('39, '39 a) have indicated the possibility that vitamin A may play an important role as a transporting agent for fatty acids across the gut wall in the halibut. Even though this may be true for certain fish, and the idea presents an interesting reason for the occurrence of large amounts of this substance in these species, the evidence at present does not constitute any substantial argument for the existence of such a mechanism in the higher mammals.

In this connection, it is of interest to point out that the occurrence of free fatty acids in the lipid extracts of intestinal mucosa has long been recognized but never satisfactorily explained. Sinclair ('28) found that as much as 36% of the acetone soluble material from the post absorptive cat mucosa was free fatty acid. During fat absorption he found somewhat lower values. About one-third of this amount could be accounted for by the acids liberated from neutral fat by intestinal hydrolysis and simply adherent to the mucosa from which it was not removed by washing. Our finding of a titratable acidity equivalent to nearly 50% free fatty acid in the total lipid from the whole intestine cannot be accounted for on this basis alone. Neither does it seem reasonable that phospholipid breakdown, with its consequent liberation of free acid, was excessive since extraction procedures such as we employed have long been used in the isolation of phospholipid. It is well known that the phospholipids are among the least stable of the tissue lipids and it has been suggested by Bloor ('24), McLean and McLean ('27) and others that a substantial part of the free fatty acid found in these extracts may have arisen from the splitting of these

compounds. Sinclair's data show that phospholipid amounts to between 35 and 45% of the total lipid of the intestinal mucosa. These values were determined as acetone insoluble fractions of the usual alcohol-ether extracts. Theoretically, fatty acids make up about 70% of the phospholipid molecule although direct analysis usually yields values between 50 and 60%. Thus, if the entire phospholipid of the intestinal mucosa were hydrolyzed during extraction, a circumstance which obviously does not occur, fatty acid from this source could not amount to more than 25% of the total lipid.

It appears to be worth noting that administered fat is absorbed from the intestine more rapidly than vitamin A since, as shown in table 1, the lipid content of the lumen becomes relatively richer in the vitamin as absorption progresses. This fact also argues against a major role for vitamin A in fat absorption. From a purely quantitative point of view, the normal occurrence of only negligible amounts of vitamin A in the gut wall of the rat (Baumann, Riising and Steenbock, '34), in comparison with relatively large amounts of fatty acid, may be considered as evidence in the same direction.

SUMMARY

Vitamin A, in the form of the naturally occurring esters of fish liver oil, has been administered to post absorptive rats and the per cent of free alcohol in the lumen and in the gut wall determined at varying time intervals using the technique of analytical distillation. Of the total vitamin A recovered, it was found that the per cent of alcohol increased from 4.36 at 80 minutes to 16.20 at 400 minutes while the alcohol in the gut wall increased from 59.2% at 220 minutes to 81.9% at 400 minutes. These data indicate that vitamin A esters behave as do other esters of the fatty acids in the intestinal tract in that they are hydrolyzed by the enzymes present there and, further, that during the height of absorption, the vitamin exists in the gut wall chiefly as the alcohol.

ACKNOWLEDGMENTS

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EFFECT OF FLUORINE ON THE ACTIVITY OF VITAMIN D IN RACHITIC RATS

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FOUR FIGURES

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The toxicology and physiology of fluorine are being studied from two somewhat different points of view: (1) as a general protoplasmic poison and (2) as a modifier of calcium and phosphorus metabolism, the latter, chiefly through its action on bone and tooth development in growing animals. Quantitatively, a great deal is now known concerning the factors which influence the healing of rickets produced in rats on high calcium-low phosphorus diets such as the no. 2965 ration of Steenbock and Black ('25). This is especially true of the amounts of vitamin D required to produce definite degrees of healing or recalcification in the rachitic metaphysis. It occurred to us, therefore, that the standard vitamin D assay technique should prove a useful tool in the study of the effects of fluorine on those phases of calcium and phosphorus metabolism involving bone formation in young growing animals.

In the experiments described herewith, we have found definite radiographic evidence that fluorine does markedly affect both the development of rickets and the healing produced by vitamin D. Furthermore, the influence of this agent on the healing process appears to depend on both duration and degree of the toxicosis which exists at the time the vitamin is administered.

EXPERIMENTAL

Three experiments will be described. The first consists of observations made incidentally in connection with another study of fluorosis (Hodge and Finn, '39). The other two were designed specifically to test the effects of fluorine on vitamin D action. All observations were made by the radiographic technique of O'Brien and Morgareidge ('38), and in the two latter experiments the young rats were taken from our standardized vitamin D assay colony.

Experiment 1. One hundred and thirty-five stock rats, taken at weaning (21 days), were divided into three groups of forty-five each. Litter mates and sexes were distributed as equally as possible among the groups. The control group was fed a basal diet which has been shown to be adequate for normal growth and bone development (Hoppert, Webber and Canniff, '31) and which has the following composition:

	Per cent
Whole yellow corn (freshly ground)	58
Whole powdered milk	30
Linseed meal	6
Alfalfa leaf meal	3
Dried yeast [1]	2
Sodium chloride	1

[1] From the Northwestern Yeast Co., Chicago, Ill.

For the first experimental group, the whole powdered milk was replaced by an equal amount of commercial casein and will be referred to as the "casein diet." This diet failed to support normal growth or bone development. The second experimental group also received the casein diet but, in addition, was given 3.0 mg. of fluorine daily in 1 drop of a water solution of potassium fluoride delivered orally from a calibrated dropping pipette. Both basal and casein diets together with distilled water for drinking were provided ad libitum.

On the fiftieth day of this regime, radiograms of the left knee-joints were made. The control group (basal diet) was found to exhibit entirely normal bone structure (fig. 1 a).

The rats on the casein diet, however, showed evidence of rickets as indicated by the fact that about 50% of the animals had moderately decalcified metaphyseal zones (fig. 1 b). Although the rats receiving the casein diet plus fluorine also exhibited a similar rachitic picture as judged by the width and density of the uncalcified metaphyses, the tibial shafts and areas of the diaphysis proximal to the rachitic zones were, if anything, more densely calcified than normal (fig. 1 c).

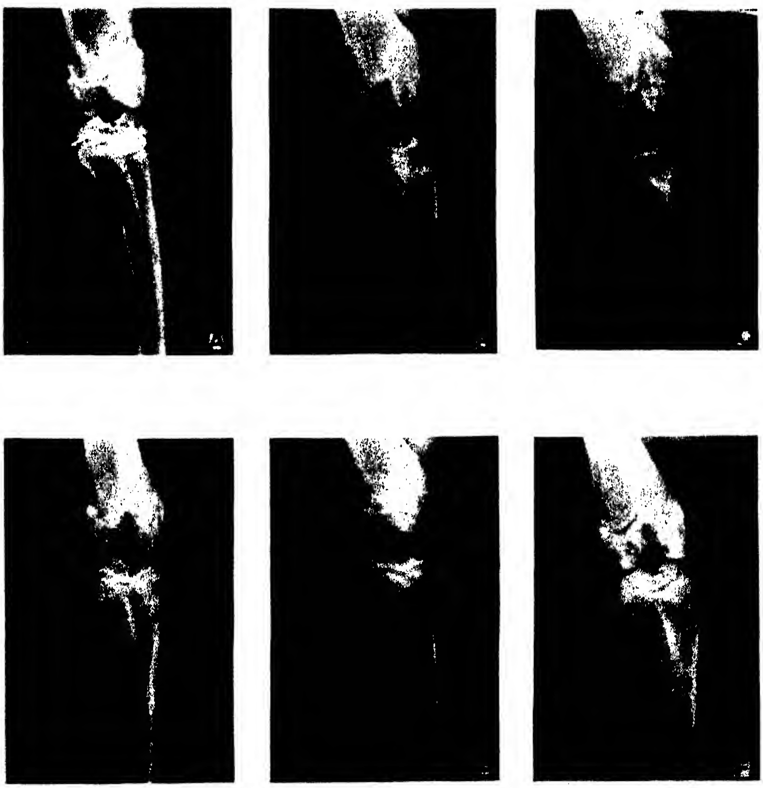

Fig. 1 A and D: Radiograms of same rat before and after addition of cod liver oil to control diet. B and E: Radiograms of same rat before and after addition of cod liver oil to the casein diet. C and F: Radiograms of same rat before and after addition of cod liver oil to the fluorine contaminated casein diet.

On the fifty-first day, 2% of cod liver oil was added to the diets of all three groups, replacing an equivalent amount of corn meal. Ten days later radiographic examination was repeated. The bones of the control group remained unchanged in appearance, i. e., they were normal (fig. 1 d). Obviously, the whole powdered milk had contained enough antirachitic activity to allow normal development. The rickets which had been observed in the animals on the casein diet had healed in a normal fashion as shown by a reduction in width of the uncalcified zone to that of the normal epiphyseal cartilage (fig. 1 e). In the group receiving the casein diet plus fluorine, however, the effect of the added vitamin D was the production of an atypical, unorganized calcification in which the epiphyseal cartilage was nearly obliterated (fig. 1 f). The effect of fluorine in this case, therefore, would appear to be that of disorganizing the bone-forming cells of the matrix without interfering markedly with the deposition of inorganic material under the stimulus of vitamin D.

Experiment 2. Three hundred parts per million of fluorine were incorporated into the Steenbock 2965 diet. This was done by thoroughly mixing a saturated solution of ammonium fluoride in 95% alcohol with the dry diet. The wet mixture was then spread in thin layers on large sheets of wrapping paper, allowing the alcohol to evaporate. Young rats, weaned at 21 days, were divided into two groups of twenty-six each. One group was placed on the fluorine contaminated 2965 diet and the other, on the same diet without the fluorine. After a rachitogenic period of 21 days, radiographs of the animals in each group were made and the type and degree of rickets compared. The results are seen in figure 2, in which the knee joints of three representative rats from the control group (top row) may be compared with those of three rats from the fluorine group (bottom row). The most obvious difference is the greater over-all density in the bones of the fluorine-fed animals. Also, the metaphyseal zone of hypertrophic cartilage is narrower in the fluorine group. Thus, as indicated by x-ray examination, a 300 p.p.m. contamination of

fluorine in the rachitogenic diet prevents the development of the usual rachitic picture.

In accordance with the usual assay procedure for vitamin D, international standard irradiated ergosterol solution, diluted in corn oil, was then administered to both groups of rats at a level of 0.5 I. U. per day for 8 days. On the tenth

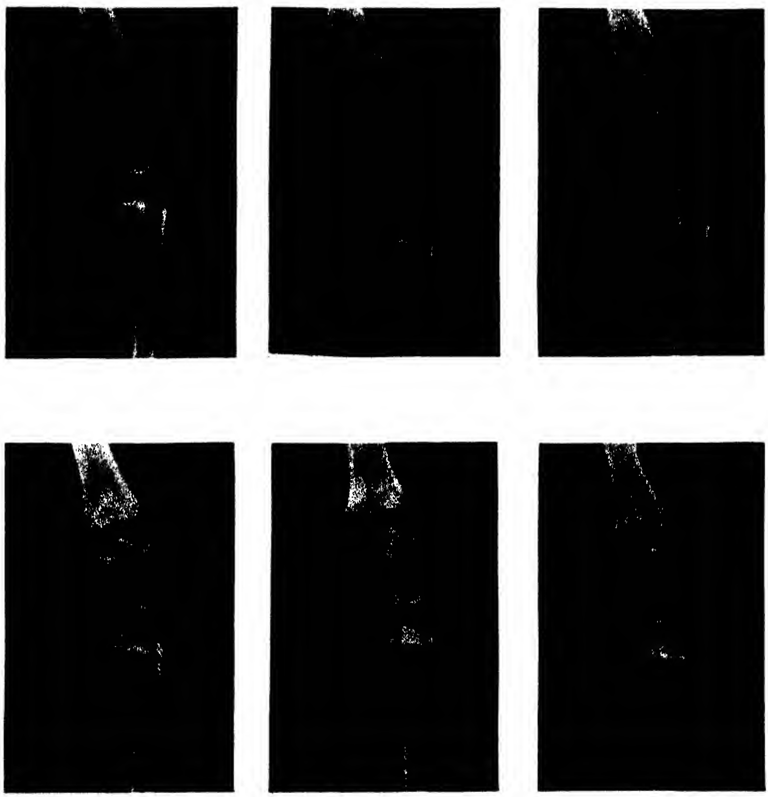

Fig. 2 Top row: Radiograms of three representative rats from the control group after 21 days on the 2965 diet showing ordinary rickets. Bottom row: Radiograms of three representative rats from the group which had received the 2965 diet contaminated with 300 p.p.m. of fluorine for 21 days. Note the abnormal metaphyseal calcification and generally increased density in this "fluorine rickets."

day, another radiographic examination was made. Figure 3 (top row) shows the appearance of the healing obtained in three representative rats from the fluorine-fed group as contrasted with that obtained in the normally rachitic animals of the control group (bottom row). Again, the effect of fluorine is striking. The new calcification in the fluorine-fed rats is diffuse and appears to be continuous with the old calcification which was previously present in the diaphyseal

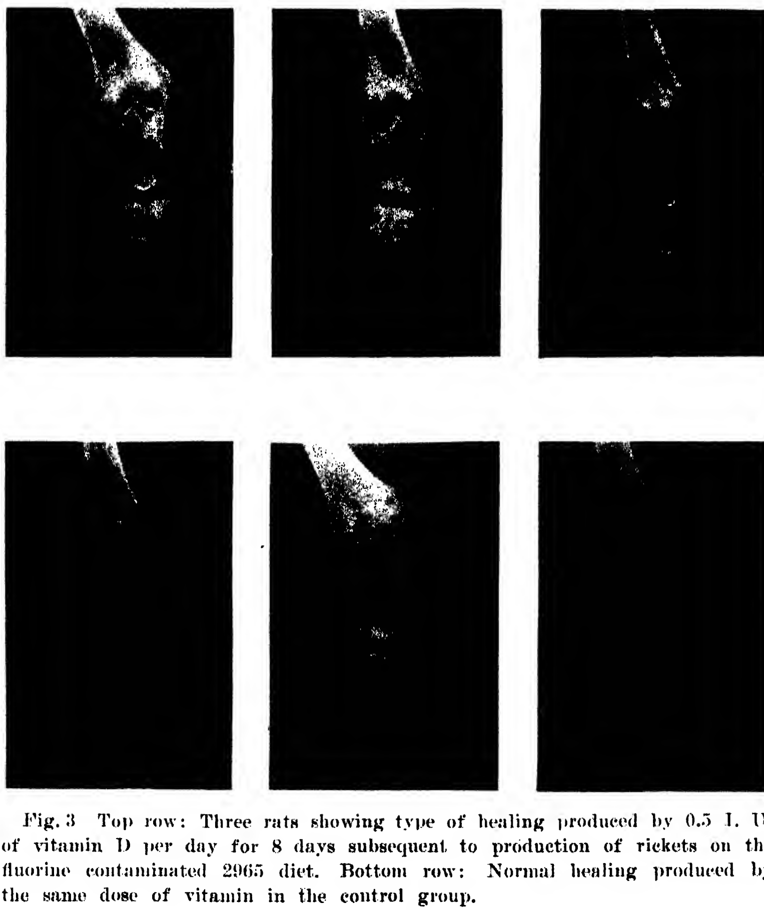

Fig. 3 Top row: Three rats showing type of healing produced by 0.5 I. U. of vitamin D per day for 8 days subsequent to production of rickets on the fluorine contaminated 2965 diet. Bottom row: Normal healing produced by the same dose of vitamin in the control group.

border. It would be extremely difficult, if not impossible, to make any quantitative estimation of vitamin D activity from such pictures. Thus, fluorine contamination of a diet from an accidental source, such as was recently reported (Hodge, Luce-Clausen and Brown, '39), would lead to severe distortion of vitamin D assay results.

Experiment 3. The results of the two preceding experiments suggested that some effect could be expected from the simultaneous administration of fluorine and vitamin D to rachitic rats which had had no previous exposure to this element. Accordingly, five groups of seven rats each were prepared for vitamin D assay by the usual 21-day period on the 2965 diet. Before starting the test feeding, radiographic examination was made to establish the fact that all had attained a uniformly satisfactory degree of rickets according to the standards set up for our routine assay work. The animals, at this time, were 42 days old and averaged between 50 and 55 gm. in weight. Each of the 35 rats was then given 0.5 I. U. of vitamin D (as the international standard solution) per day for 8 days. One group was kept for control and the other four given graded doses of fluorine in 1 drop of aqueous potassium fluoride solution, daily. The levels of fluorine fed were 0.5, 1.0, 2.0, and 3.0 mg., respectively, for the four groups. Final radiograms were made on the tenth day and the degree of healing found in the fluorine-fed animals compared with that of the controls. It was found that the curative power of the vitamin had been definitely inhibited by the fluorine and that the amount of inhibition was roughly proportional to the dose given. The healing produced by the half unit of vitamin D plus 3.0 mg. of fluorine was only 50% of that resulting from the same dose of vitamin alone. Similarly, the 0.5 mg. dose of fluorine resulted in about a 10% inhibition in healing.

The "line" of new calcification seen in these animals was quite typical of normal healing produced by vitamin D. The doses of fluorine used over the 10-day period of the experiment were not sufficient to produce symptoms of toxicity.

The diffuse, unorganized deposition of bone salt seen in the two previous experiments was absent. The radiograms in figure 4 (top row) show three representative rats from the control group which received 0.5 I. U. of vitamin per day with no fluorine. Figure 4 (bottom row) shows three rats from the group given 3.0 mg. of fluorine and the same dose of vitamin D. The marked reduction in healing caused by fluorine is evident.

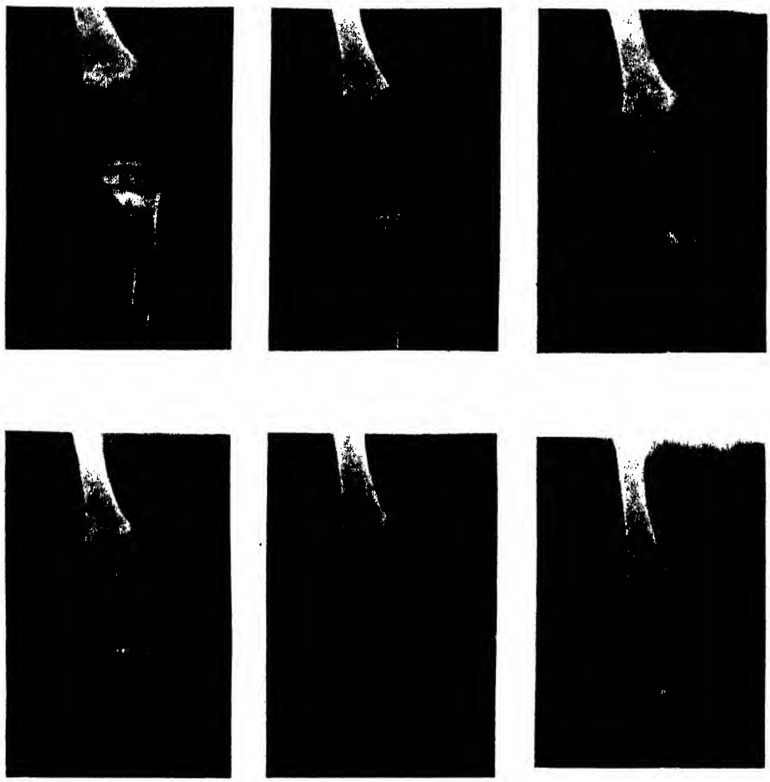

Fig. 4 Top row: Normal healing produced in rachitic rats by 0.5 I. U. of vitamin D per day for 8 days. Bottom row: Healing produced by the same amount of vitamin D administered simultaneously with 3.0 mg. of F per day. These animals were not exposed to fluorine during the rachitogenic period. Note the reduction in the amount of healing.

DISCUSSION

Hauck, Steenbock and Parsons ('33) added 0.15% fluorine to the 2965 diet and found an increase in total bone ash over that of control rats on the same diet without fluorine. Their experimental period was 6 weeks. When vitamin D was also incorporated into the fluorine-containing ration, they obtained a slightly higher ash content of the bone over that produced by vitamin D alone. Our radiographic evidence, especially in experiment 2, confirms their findings and shows, in addition, that the greatest concentration of this extra calcification accumulates near the diaphyseal border of the metaphysis. It is of interest to note that the authors quoted above also found that in diets with a normal Ca:P ratio, the addition of fluorine failed to increase the ash content of the bones.

Our experiment 3, however, indicates that a distinction must be made between the chronic and acute types of fluorosis as regards their effect on vitamin D. The fact that fluorine has the power to inhibit the classical picture of healing rickets suggests that further experiments of this type may shed some additional light on the mode of action of vitamin D.

SUMMARY

Summarizing, we have found: (1) In rats on a rachitogenic diet containing fluorine, there appears to be a decrease in the severity of the rickets due to a general increase in the density of the bones to x-rays and, more particularly, to a decrease in width of the metaphyseal zone of hypertrophic cartilage. (2) Vitamin D promotes the healing of this "fluorine rickets" but the deposition of new bone salt is atypical, its appearance suggesting that fluorine has in some way interfered with the orderly arrangement of the matrix cells. (3) Fluorine inhibits the healing process when given simultaneously with vitamin D to rachitic rats which have not been previously subjected to fluorine poisoning. This inhibition is roughly proportional to the dose given and amounts to 50% at a level of 3.0 mg. of fluorine per day.

ACKNOWLEDGMENTS

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EDITORIAL REVIEW

MANGANESE, LEAD, TIN, ALUMINUM, COPPER, AND SILVER IN NORMAL BIOLOGICAL MATERIAL

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The spectrochemical data on manganese, lead, tin, aluminum, copper, and silver in the diet, the tissues, and the excreta of man given in a recent article (Kehoe et al., '40) were obtained primarily as a basis for our future investigations, but since, of necessity, they contradict, confirm, or supplement the observations of others it is our present purpose to review the more recent work and to summarize the pertinent facts concerning the normal occurrence of these metals in biological material.

MANGANESE

Manganese might be expected to occur generally in biological material, considering its recognized physiologic usefulness (Langecker, '34; von Oettingen, '35) and this expectation has been confirmed by many observations. Comparatively large quantities (approximately 4 mg. per day) are found in the human diet and substantially equivalent amounts are eliminated in the excreta (Kehoe et al., '40). The chief source of manganese in the diet, as shown by Boycott and Cameron ('30), Remington and Shiver ('30), Richards ('30), and Peterson and Skinner ('31), is of vegetable origin, cereals being particularly rich in this metal (Davidson, '29; Richards, '30).

The regular occurrence of manganese in the tissues of animals has been demonstrated among others by Bertrand and Ciurea ('31), Reiman and Minot ('20), Turnwald and Haurowitz ('29) and Chevalier ('30). The latter found low concentrations of manganese in the organs and noted that there was

not much variation in the average concentration in most tissues (0.03 to 0.04 mg. per 100 gm. of fresh tissue). Our data ('40) agree with these observations and particularly with those of Reiman and Minot ('20) who reported values of 0.17, 0.061, 0.035 and 0.036 mg. per 100 gm. of liver, kidney, brain, and stomach, respectively. Information on the distribution of manganese in the skeleton is scanty and our results of 0.17–0.30 mg. per 100 gm. of human bone ('40) are somewhat higher than the mean of 0.101 mg. per 100 gm. of the bones of rabbits, cats and dogs reported by Lund, Shaw and Drinker ('21).

Bertrand and Medigreceanu ('13), Reiman and Minot ('20), Abderhalden and Möller ('28), Dutoit and Zbinden ('29) and Adam and Horner ('37) found manganese in the blood of normal animals regularly. With the exception of one result of 1.15 mg. per 100 ml. in horse serum (Abderhalden and Möller, '28), low concentrations have been found in the blood. Bertrand and Medigreceanu ('13) found a higher concentration of manganese in the blood of the sheep than in that of any other animal investigated. Their value of 0.002 mg. per 100 ml. of human blood is considerably lower than the mean of 0.014 mg. per 100 ml. calculated from Reiman and Minot's data ('20) and our mean of 0.015 mg. per 100 ml. ('40). Practically all of the manganese in human blood is found in the cells (Kehoe et al., '40), but Bertrand and Medigreceanu ('13), in the case of sheep blood, found approximately two-thirds in the plasma. Our results revealed slight but definite variations in the manganese concentration of repeated samples from the same individual (Kehoe et al., '40), in contradiction of Reiman and Minot's ('20) observations that the level of concentration was constant for each individual.

McCrackan and Passamaneck ('26) failed to find manganese in the urine of normal individuals since it was not present in concentrations of 0.02 mg. or more per liter. Probst ('33) using flame spectra also was unable to detect manganese in the urine even in patients treated with psorimangan (a proprietary colloidal manganese preparation). On the other hand our data ('40) indicate that manganese is always present in amounts

somewhat less than 0.01 mg. per liter. Probst's ('33) failure to detect it may be attributed to the lower sensitivity of the flame as compared to the arc method of excitation.

COPPER

Like manganese, copper is physiologically important and is apparently a constituent of practically all plant and animal tissues (Eichholtz, '34; Gerlach, '34; Elvehjem, '35; Hahn and Fairman, '36; Eggleton, '38). Since the detection of copper in blood by Sarzeau (1830), the distribution of this metal in the animal organism has been the subject of exhaustive study, but there is still some disagreement as to its range of concentration in certain tissues. This is especially true in the case of blood, in which concentrations from 0.05 to 0.28 mg. per 100 ml. have been reported (Tompsett, '34 a). Tompsett's ('34 a) figures of 0.185 to 0.229 mg. per 100 ml. are considered too high by Elvehjem ('35), while Sachs and his co-workers ('35) obtained values of 0.141 and 0.132 by two different methods. Guillemet ('32) found 0.088 mg. per 100 ml. of beef blood, and Elvehjem and his collaborators ('29) approximately 0.05 mg. per 100 ml. of horse blood. The results of Schönheimer and Oshima ('29) (0.113 to 0.14 mg. per 100 ml. of human blood), and the average value of 0.098 reported by Bence and collaborators ('36), as well as the range of 0.091–0.108 given by Sarata ('35), are not far from our figure of 0.114 ('40). There is also some disagreement as to the distribution of copper between cells and plasma of blood. Bjerrum and Henriques ('35) found that the copper in rabbit blood was equally distributed between cells and plasma; Tompsett ('34 a) reported like results for human blood, but Guillemet ('32) found more copper in the plasma than in the cells of dog, pig, and ox blood. On the other hand, Elvehjem and co-workers ('29) reported that the cells of horse blood contained more copper than the plasma, and Sarata ('35) has given values of 0.034–0.064 mg. in plasma and 0.068–0.073 mg. in the erythrocytes. Our data ('40) generally confirm the latter figures but reveal the occurrence of exceptions to the rule that the concentration in the erythro-

cytes exceeds that in the plasma. The degree of variability in the copper content of the blood fractions from the same individual has not been determined, but it has been shown that the concentration in the whole blood is not constant (Kehoe et al., '40).

Our results on the copper concentration in human tissues ('40) are in general agreement with those on normal animal tissues reported by Bodansky ('21), Chou and Adolph ('35), Tompsett ('35), and Hahn and Fairman ('36). The relatively large quantities of copper found in the liver and brain are within the variations of 0.30 to 0.91 mg. per 100 gm. of liver and 0.22 to 0.46 mg. per 100 gm. of brain noted by Tompsett ('35), and 0.36 to 0.68 mg. per 100 gm. of brain found by Bodansky ('21). Comparatively large quantities of copper have also been found in bone. Tompsett ('35) gave results varying from 0.37 to 0.47 mg. per 100 gm. of rib, in conformity with our findings ('40). Tompsett did not analyze the long bones, and, except for our figure of 1.19 mg. per 100 gm. of such bones and for the report of Tiede and Chomse ('34) that copper is a natural constituent of teeth (1.2 mg. per 100 gm. of bovine teeth), we have not found additional data on bony structures.

The available data agree as to the daily ingestion and excretion of copper. The mean daily intake and output has been put at approximately 2 mg. per day by Chou and Adolph ('35), Tompsett ('35), Leverton ('39), and ourselves ('40). As in the case of manganese, most of the ingested copper is eliminated by the alimentary canal and only small quantities appear in the urine. Chou and Adolph ('35) report an average daily urinary output of 0.25 mg. per day, and Tompsett ('34 b) sets the average concentration at 0.18 mg. per liter. Leverton ('39) has recently reported variations in daily output among young women ranging from 0.025 to 0.325 mg., while Rabinowitch ('33) found concentrations varying from traces (ten instances) to 0.41 mg. per liter in the urine of fifty normal persons. Ross and Rabinowitch ('35) noted a similar range of variation in the urine of normal children. These results are considerably higher than ours ('40), which, for the most part, correspond

only to the lower range of the values given by Leverton ('39) and Rabinowitch ('33), a fact which raises the question of contamination in the case of the higher figures.

ALUMINUM

The effects of aluminum on the human organisms have been investigated extensively, and it is apparently established that the daily ingestion of amounts up to 200 mg. aluminum per day is harmless (U. S. Dept. Agr., '14; Burn, '32; Monier-Williams, '35). Sources of aluminum ingestion other than from cooking utensils and certain baking powders are found in foods of vegetable and animal origin as shown by Kahlenberg and Closs ('29), Underhill and Peterman ('29 b), Underhill et al. ('29), Yoshii and Jimbo ('32), Wührer ('33), Meunier ('36), and Webb ('37). Little of the ingested aluminum is absorbed, and in conformity with the findings of Underhill and Peterman ('29 a), Mackenzie ('30, '31), and Wührer ('33), our data ('40) show a practical equivalence between the amounts excreted daily in the feces and those occurring daily in the food.

The occurrence of aluminum in human tissues has been the subject of controversy (McCollum et al., '28, '30; Kahlenberg and Closs, '29). Our observations ('40) confirm the conclusions of the majority of investigators that aluminum is regularly present in small amounts. They are in excellent agreement with those of Myers and Mull on the normal tissues of man ('28 b), dog, and rat ('28 a), and with those of Wührer ('33) on the tissues of the dog.

Except for the results on two samples of dog blood given by Eveleth and Myers ('36), our mean concentration of 0.013 mg. per 100 ml. of blood (Kehoe et al., '40) is considerably lower than other recorded values. The values reported by Underhill and Peterman ('29 b) and by Mull and his co-workers ('27) approach 0.20 mg. per 100 ml. of blood. The latter investigators obtained lower values when larger samples were used, a fact highly suggestive of some factor of contamination. The carriage of almost all of the aluminum of the blood in the plasma and not in the formed elements, as shown by our

observations (Kehoe et al., '40), has apparently not been reported by others.

TIN

In agreement with our data ('40) tin has been found irregularly in normal biological material by a number of investigators. Newell and McCollum ('31) and Webb ('37) found tin in certain marine products while Boyd and De ('33) detected it in human tissues and in occasional samples of vegetable material. Staud ('36) found it in canned beer, and Dingle and Sheldon ('38) noted its occurrence in processed milk of human and other origin. Tin was present in very small quantities in about 80% of our samples ('40). When found in blood, practically all of the tin was in the cells (Kehoe et al., '40). It occurred in appreciable quantities in the food and feces of normal American subjects and, as in the case of the other metals referred to herein, there was an approximate balance between the amounts excreted in the feces and the quantities ingested with the food (Salant et al., '14; Kehoe et al., '40).

Several investigators have regarded tin as a regular constituent of certain biological materials. Bertrand and Ciurea ('31) found it in nearly all organs of the ox, the horse and the sheep, the quantities varying from 0.05 to 0.40 mg. per 100 gm. for all dry tissues except the skin, hair and tongue. According to Schübel ('34), the quantities found in the muscle and mucous membrane of the tongue were so high, relatively, as to have some specific functional significance, but no such role has been defined. Misk ('23) accepted tin as a normal constituent of human viscera, and believed that the human body contained as much tin as zinc. Dutoit and Zbinden ('30) also found tin in all organs, and especially abundantly in the brain, spleen, and thyroid. In disagreement with their results on the brain we encountered lower concentrations of tin in this tissue than in any other (Kehoe et al., '40). Jost ('34) and Lowater and Murray ('37) found tin in all teeth examined while Scott and McMillen ('36) reported its regular presence in spinal fluid.

SILVER

Only 10% of the samples of human tissue which we analyzed contained silver, and then in concentrations which were rarely greater than 0.01 mg. per 100 gm. ('40). Larger quantities were found only in feces and prepared food samples, these, no doubt, resulting from the use of silver tableware. The quantities found in the feces and food were smaller than those of the other metals studied (0.06–0.08 mg. per day) but in this case also the balance between the quantity eliminated daily in the feces and that ingested daily in the food was maintained (Kehoe et al., '40).

This same irregularity in the occurrence of silver in normal biological material was noted by Dutoit and Zbinden ('30), Fox and Ramage ('30, '31), Newell and McCollum ('31), Boyd and De ('33), Ramage, Sheldon and Sheldon ('33), Rusoff and Gaddum ('38), Kogan and Nasuirova ('35) and Lowater and Murray ('37).

LEAD

Our observations on the occurrence of lead in the tissues and excreta of normal human beings, up to 1933, together with a review of the results of other workers, have been detailed elsewhere (Kehoe et al., '33 a, '33 b, '33 d). Recently published data (Kehoe et al., '40) have provided further evidence of the correctness of our statement that "the presence of lead in human excreta and human tissues, as well as in living organisms in general, is an inevitable consequence of life on a lead bearing planet" (Kehoe et al., '33 a).

The freely chosen human diet varies considerably in its lead content from day to day (Kehoe et al., '40), and, as we have pointed out (Kehoe et al., '33 c), the increment of lead content above that which is present naturally is due to contamination from a great variety of sources. These sources have also been discussed by Monier-Williams ('38) whose data on the natural lead content of certain foods are in general agreement with our own. Observations on a series of subjects have shown that the mean daily lead intake in the diet is approximately

equivalent to the mean daily output of lead in the feces, it being about 0.30 mg. per day (Kehoe et al., '33 b, '35, '40).

The mean concentrations of lead in normal human tissues which we have reported (Kehoe et al., '33 b, '40) are in close agreement with those obtained in recent years by other investigators (Barth, '31; Weyrauch and Müller, '33; Lynch et al., '34; Tompsett and Anderson, '35; Tompsett, '36; Pernice, '36, and Bagchi et al., '39). The extent of the variation in the gross quantity of lead in the normal adult human body, and especially in the skeleton, is somewhat questionable. Barth's data ('31), in his opinion, pointed to a progressive increase in skeletal lead with age, and Calvery ('38) has made a similar interpretation of Tompsett's data ('36) on the lead content of the femur in a series of cases. Bagchi et al. ('39) did not find concentrations of lead in normal human bones in the higher range of Tompsett's values, nor have we obtained such results except in persons with definite histories of lead exposure. Our data up to the present do not demonstrate any relationship between lead concentration in the skeleton and age ('40).

All recent investigators are in agreement as to the normal occurrence of lead in human blood, the lowest values (with an upper limit at 0.04 mg. per 100 ml. of blood) having been given by Litzner and Weyrauch ('32, '33). Teisinger ('36) has set the mean concentration at 0.061 mg. per 100 ml., Tompsett and Anderson ('35) have given it as 0.055 mg., while Smith and co-workers ('38) found variations between 0.01 to 0.05 mg. per 100 cc. of normal blood. The data of Taeger and Schmitt ('37) derived from the analysis of the blood of 148 normal persons yield a mean concentration of 0.035 mg. per 100 cc. of blood, while Willoughby and Wilkins ('38) obtained a mean of 0.025 mg. per 100 cc. of blood in 189 normal cases. The mean value of our earlier results on seventy-one normal young men was 0.058 mg. per 100 gm. of blood (Kehoe et al., '35), but refinements in analytical technique and, more importantly,

rigid precautions in collecting and handling samples have reduced the mean of our results (Kehoe et al., '40) to a point which coincides with that of Willoughby and Wilkins, individual values ranging from 0.01 to 0.06 mg. per 100 gm. of whole blood.

In determinations of the distribution of lead in the blood fractions Willoughby and Wilkins ('38) obtained negative results in approximately 90% of fifty-eight samples of blood serum, and only insignificant quantities in the remaining samples. Smith and co-workers ('38) also failed to find lead in normal serum, while Teisinger ('36) found only minute quantities in the serum. The latter also studied the distribution between the plasma and cells and reported that the lead was equally divided between the two. This is in disagreement with our findings (Kehoe et al., '40) which clearly indicate that the normal plasma is almost free of lead. The results on twenty-seven samples of normal human plasma ranged from 0 to 20% of the total lead in the blood, twenty-three yielding less than 10%.

Schmitt and Basse ('38) studying the diurnal variations in the concentration of lead in the blood of five normal persons found elevated concentrations during the early morning hours. Our corresponding observations on the blood of normal persons (which with corresponding urinary findings will be reported elsewhere) have failed to show significant variations during the 24-hour period.

The mean values established for the lead concentration in normal urine (approximately 0.030 mg. per liter) (Kehoe et al., '35, '36, '40) are in general correspondence with the results of Bass ('33), and Behrens and Taeger ('35) in Germany, Ross and Lucas ('35) in Canada, Boyd and Ganguly ('32) in India, Reith and van Dijk ('38) in Holland and Tompsett and Anderson ('35) in England, if due allowance is made for variations in the sensitivity and accuracy of the analytical methods employed by these investigators.

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ASCORBIC ACID CONTENT OF GOAT'S MILK AND BLOOD: INFLUENCE OF ASCORBIC ACID INJECTION AND DIET[1]

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THREE FIGURES

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Hunt and Winter ('22) found that 20 to 30 cc. of goat's milk daily protected guinea pigs from scurvy; their bio-assays indicated that goat's milk contained somewhat more vitamin C than cow's milk. Meyer and Nassau ('24) and Frank ('25) reported the contrary result, while Ruijter de Wildt and Brouwer ('30) found approximate equality in the vitamin C content of milks of the two species. Chakraborty ('35) found in five samples of goat's milk from 0.7 to 1.1 mg. of ascorbic acid per 100 gm. Rudra ('36) found 8.5 mg. per 100 cc. Cimmino ('38) found a mean value of 4.5 mg. per 100 cc. for four samples. These results are all based on bio-assays or on the titration of a small number of samples.

Since the commencement of our study an extensive report has been made by Gamble, Ellis and Besley ('39) on the composition of goats' milk. Vitamin C was determined by bio-assays during the years 1929–30–31, supplemented by chemical determinations at monthly intervals during the period June,

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1936 to June, 1937. The handling of the milk for the later chemical tests was kept as nearly as possible the same as for the earlier bio-assays. The method of estimation was to precipitate the milk proteins with trichloracetic acid, filter, and titrate the filtrate with 2,6-dichlorophenol-indophenol. Individual samples contained from 0.5 to 2.5 mg. of ascorbic acid, with a mean of 1.3 mg., per 100 cc. of milk. Spring and summer milks appeared to have a slightly higher titre than fall and winter milks. It was suggested that the relative abundance of green forage was probably the most important factor determining the variations.

Sharp ('36) stated that the vitamin C content of fresh milk is not increased by the daily intrajugular injection of 4 gm. vitamin C in the goat.

Wendt ('38) reported that the ascorbic acid content of cow's milk had a relatively constant value of about 20 mg. per liter and that it was unaffected by the injection of vitamin C.

On the other hand, Rasmussen, Bogart and Maynard ('38) found temporary rises in ascorbic acid content of ewe's milk and of cow's milk within 14 hours after the intravenous injection of ascorbic acid. The increase amounted to approximately 50% of the initial values.

EXPERIMENTAL

Animals used. Two groups of goats were used in this work, the first consisting of five Toggenburgs and the second of four assorted goats. The pertinent history of the Toggenburgs is given below:

GOAT	BORN	KIDDED	REMARKS
794	5/25/32	6/ 6/38	
906	2/ 9/33	5/ 2/38	
1000	2/22/33	5/12/38	
10	5/20/37	6/17/38	Kid of no. 1000
11	5/20/37	7/ 9/38	Kid of no. 1000

Until 11/10/38 these goats were kept in a pen with their kids and were not milked regularly. On 11/10/38 they were put into individual stalls and then milked twice daily. On 4/25/39 they were turned out to pasture. Their daily ration consisted

of good quality hay (alfalfa hay until 2/14/39 and then lespedeza hay) and a grain mixture of 4 parts white corn, 3 parts cottonseed meal, 2 parts wheat bran and 1 part oats. Occasionally the grain mixture was replaced by oats alone.

On three occasions they were given injections of ascorbic acid [3] as follows: 1 gm. each on 2/21/39, 1 gm. each on 4/17/39 and 2 gm. each on 5/17/39. Each dose was dissolved in reboiled distilled water and injected intraperitoneally about 11 A.M. Milk samples were collected just before the injections, about 4 hours later, twice the following day, and then once daily in the morning until the effect of the injection was no longer noticed. Blood and urine samples were collected at some of these milking-times.

The second group of goats was as follows:

GOAT	KIDDED	BREED	ON C-FREE DIET
3	3/14/39	Angora	5/20/39 to 7/ 1/39
8	3/19/39	Half Toggenburg	7/ 3/39 to 9/30/39
M 1	2/18/38	Half Toggenburg	4/19/39 to 7/ 1/39
M2	3/10/39	Half Toggenburg	4/19/39 to 5/20/39 and 7/ 1/39 to 9/30/39

Except during periods on a vitamin C-free diet these goats were kept in a pen with their kids and fed hay only until 5/10/39. On this date they were turned out to pasture with the Toggenburgs and given the same hay and grain ration. On account of a snagged shoulder, M 2 was kept in an outside pen from 6/12/39 to 7/1/39 and fed hay only. When kept on the vitamin C-free diet the goats were penned in a small house having a concrete floor covered with sawdust. The periods spent in this pen are shown above; the only food given was the grain mixture.

PROCEDURE

Blood and milk samples were taken from each goat at approximately weekly intervals except between 10/15/38 and 2/2/39 when milk samples were taken twice weekly. The blood was drawn from the jugular vein. Samples were taken in the

[3] Our thanks are due to Merck and Company for the gift of the ascorbic acid used in this study.

morning and kept in an electrical refrigerator in dark glass bottles until titrated; bottles for the blood contained 20 mg. sodium oxalate and 10 mg. potassium cyanide per 10 cc. of blood.

The method of titrating milk and colostrum samples was essentially that of King ('37). Five cubic centimeters of milk was added to 3 cc. of a solution containing 8% acetic acid and 4% metaphosphoric acid in a 40 cc. capsule vial. This was titrated against 2,6-dichlorophenol-indophenol, using a modified Neale-Forbes titration assembly and adding the dye solution until one drop produced a faint pink visible for 30 seconds. Each titration was completed within 3 minutes. The dye solutions were standardized in a manner similar to that described by Tripp, Satterfield and Holmes ('37) at such a concentration that about 0.3 to 0.4 cc. was required for each titration. On account of high viscosity, colostrum samples were usually treated with more than 3 cc. of the acid mixture.

Vitamin C in blood was determined by a method found to be the most reliable in a series of comparative studies by Satterfield, Perlzweig and Dann ('37). Four cubic centimeters of plasma, which must be free from hemoglobin pigments, was mixed with 6 cc. of reboiled distilled water and 10 cc. of 4% metaphosphoric acid and filtered. Five cubic centimeter portions of the filtrate were titrated as above, with a dye equivalent to approximately 0.02 mg. ascorbic acid per cubic centimeter.

Several hundred milk and blood samples were titrated, and the individual results plotted. The graphs for one of the Toggenburgs and each of the other goats are reproduced in the figures.

RESULTS

The complete data showing variation in ascorbic acid content of the blood and milk under normal conditions and the effect of injecting ascorbic acid are given for only one of the five Toggenburg goats because they are typical of the group (fig. 1). The range and mean values of the ascorbic acid content of the milk and blood of each of the five goats are given in table 1.

The mean value of ascorbic acid content of all milk samples, excepting those obtained within 2 weeks after injection, was 1.24 mg. per 100 cc.; and about 90% of the samples fell within the range 0.5 to 2.0 mg. per 100 cc. These figures agree well with the values obtained by Gamble, Ellis and Besley quoted

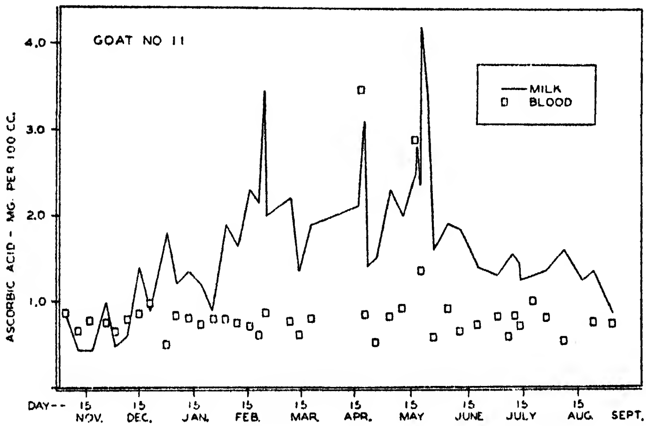

Fig. 1 Vitamin C content of the milk and blood of a typical Toggenburg goat under normal conditions and after injection of 1 gm. ascorbic acid on 2/21 and 4/17 and 2 gm. on 5/17. Values obtained on 5/20 and 5/21 and given in table 3 are omitted because of the small scale of the figure.

TABLE 1

Ascorbic acid content of Toggenburg milk and blood in milligrams per 100 cc.

GOAT		NORMAL			AFTER INJECTION OF ASCORBIC ACID
		Number of samples	Mean ascorbic acid	Standard deviation	Maximum ascorbic acid
	11	54	1.43	0.59	4.18
	10	53	1.36	0.74	4.02
Milk	1000	53	1.30	0.58	4.53
	794	53	0.87	0.36	2.16
	906	53	1.16	0.73	3.03
	11	30	0.77	0.10	3.46
	10	29	0.80	0.13	2.99
Blood	1000	29	0.69	0.11	3.06
	794	29	0.56	0.11	2.47
	906	30	0.71	0.11	3.76

above. On the average they appear to be lower than the corresponding figures for cows' milk. Holmes, Tripp, Woelffer and Satterfield ('39) reported the mean values: for Guernsey cows 2.05, and for Holsteins 1.82 mg. per 100 cc. A survey of the literature shows that values for fresh cows' milk, protected from oxidation and irradiation, lie generally between 1.0 and 3.0 mg. per 100 cc.

Figure 1 shows the considerable variability of the ascorbic acid content of the milk under normal conditions, and the lack of close relationship between the concentrations in samples

TABLE 2

Daily average milk production (pounds) of Toggenburg goats

GOAT	NOV.	DEC.	JAN.	FEB.	MAR.	APR.	MAY
11	3.1	2.4	2.3	1.9	2.1	2.1	2.8
10	1.9	1.9	1.8	1.5	1.8	2.0	2.6
1000	3.4	3.4	3.6	2.8	3.3	2.8	3.4
794	2.1	1.8	1.6	1.4	1.6	1.6	2.0
906	2.1	1.8	1.4	1.1	1.3	0.9	0.8

of blood and milk taken at the same time. As an extreme example of the variation of concentration the following figures for no. 10 may be cited:

DATE	7/11		7/12		7/13
	a.m.	p.m.	a.m.	p.m.	a.m.
Mg. ascorbic acid per 100 cc. milk	1.34	2.22	0.98	1.60	0.71

The milk of four of the goats showed a small but definite increase over the period from November to February. A record was kept of the milk production of these goats for 7 months, and it was found that the yield varied widely between the different goats, and considerably for each goat from day to day. The variation was as great as that of the ascorbic acid concentration; sometimes in the same direction and at others in the opposite direction. The mean daily milk production for each month is given in table 2.

The effect of injection of ascorbic acid was first tested on 2/21/39 when each goat was given 1 gm. intraperitoneally. Blood samples were taken just before and 2 days after injec-

tion, so that the large temporary rise noticed after later injections was missed. The effect on the milk of the different animals was irregular at this test, as the next samples of two of the goats contained less ascorbic acid than samples taken just before injection. Examination of the urine showed a very large rise in ascorbic acid content within 4 hours after injection

TABLE 3

Milligrams ascorbic acid per 100 cc. milk, blood and urine before and after injecting each goat with 2 gm. ascorbic acid

DATE	5/10	5/17 a.m.	5/17 p.m.	5/18 a.m.	5/18 p.m.	5/19	5/20	5/21	5/22	5/26 a.m.	5/26 p.m.	6/3
MILK OF												
11	2.02	2.46 [1]	2.81	2.44	2.38	*4.18* [2]	2.98	2.66	3.28	1.62	1.48	1.96
10	1.91	2.69	2.82	2.27	2.17	3.79	*4.02*	2.75	3.40	1.06		2.03
1000	1.39	1.90	1.90	1.71	1.85	*3.28*	3.06	2.07	2.68	1.45		1.45
794	1.19	1.17	1.15	0.95	0.82	1.27	1.84	1.81	*2.16*	0.80		0.81
906	2.61	0.39	0.80	0.57	0.38	0.67	1.17	1.49	*2.38*		0.75	1.15
BLOOD OF												
11	0.920		2.87			1.28				0.592		0.905
10	0.766		1.08			1.23				0.712		0.890
1000	0.743		3.06			0.937				0.342		0.635
794	0.609		2.47			0.962				0.402		0.548
906	0.502		3.76			0.941					0.496	0.912
URINE OF												
11			601	8.2								
10			1.88	0.3								
1000			112	5.8								
794			136	0.5								
906			241	3.0								

[1] Injections were made immediately after these samples were collected.
[2] The italic figures were the maximum values observed after injection.

and a return to normal next morning. Following the second injection of each goat with 1 gm. of ascorbic acid on 4/17/39, it was found that after 4 hours the concentration in the blood was from two to five times as great as it was before injection, and that it returned to normal in about 2 days. In the milk the concentration rose between 50% and 100% for each of the goats.

The third injections, of 2 gm. ascorbic acid for each goat, were given on 5/17/39, and the complete data for the period immediately afterward are given in table 3. The next samples of blood drawn contained a greatly increased amount of ascorbic acid and the urine samples showed rapid excretion of the ascorbic acid, except for no. 10, where no significant change in

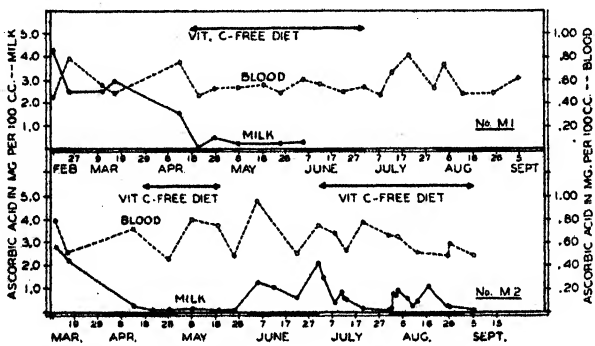

Fig. 2 Effect of diet on the vitamin C content of the milk and blood of two grade Toggenburg goats. Except for the vitamin C-free periods indicated, these goats received only hay until 5/10, after which they were turned on pasture and fed hay and grain.

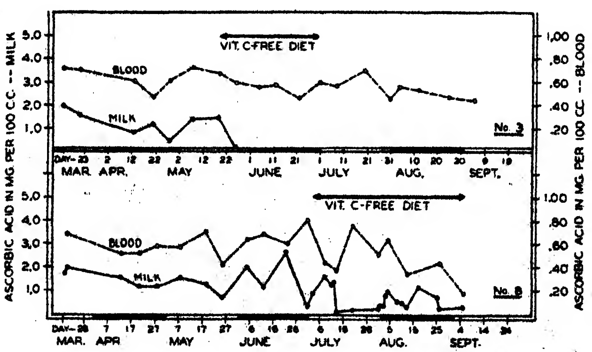

Fig. 3 Effect of diet on the vitamin C content of the milk and blood of an Angora goat (no. 3) and a grade Toggenburg (no. 8). Except for the vitamin C-free periods indicated, these goats received only hay until 5/10, after which they were turned on pasture and fed hay and grain.

blood or urine occurred. It is feared that no. 10 was not injected intraperitoneally, as this goat exhibited a large increase in blood ascorbic acid following injection of 1 gm. on 4/17/39. At the time of injection on 5/17/39, her rumen was very distended and the injected ascorbic acid may have gone into connective tissue instead of entering the peritoneal cavity.

The observations on the remaining goats, which were fed alternately a normal diet and a vitamin C-free diet are set out in figures 2 and 3. For each animal the observations began immediately after birth of a kid and commencement of a lactation period. The level of ascorbic acid in the blood is clearly not significantly affected by the change from pasture to vitamin C-free diet or vice versa. Any slight change which may have occurred is less than the day-to-day variation. This would suggest that the goat is independent of a supply of the vitamin in its diet and is able to synthesize its own ascorbic acid unless its requirement is relatively very small. The milk of some goats may possibly contain more ascorbic acid when the animals are at pasture than when they are receiving a vitamin C-free diet, as the data for M 2 and 8 suggested; but again there is no regular close relationship between the ascorbic acid content of the milk and that of the food in the animals under observation.

SUMMARY

1. From October, 1938 to August, 1939, 360 milk samples and 190 blood samples were taken from five Toggenburg goats receiving a normal diet and titrated for ascorbic acid content.

2. In blood the average ascorbic acid content ranged between 0.6 and 0.8 mg. per 100 cc., and in milk between 0.5 and 2.0 mg. per 100 cc. There was no consistent relationship between the content of the milk and that of the blood.

3. Following injections of 1 or 2 gm. of ascorbic acid intraperitoneally a rapid large rise in blood ascorbic acid, a rapid very large rise in urine ascorbic acid and a slower small rise in milk ascorbic acid were observed.

4. Observations on four other goats fed alternately a normal and a vitamin C-free diet indicated that the ascorbic acid of

the blood is not closely dependent on the amount of ascorbic acid in the diet, suggesting that the goat is independent of a dietary supply of ascorbic acid. The diet probably affects the ascorbic acid content of the milk more than that of the blood.

ACKNOWLEDGMENT

We wish to thank James L. Moore of the Department of Dairy Research for collecting the samples.

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THE EFFECT OF COMPLEMENTING FACTORS ON THE QUANTITATIVE RESPONSE AND SPECIFICITY OF VITAMIN B_6[1]

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THREE CHARTS AND ONE PLATE (SEVEN FIGURES)

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Confusing and erratic animal responses encountered in studying the interrelationship and specificity of vitamin entities have been particularly prevalent in respect to those factors concerned with dermal pathology. The characteristic skin and peripheral lesions attributable to vitamin B_6 deficiency are seemingly difficult to obtain or reproduce with consistency in some laboratories; furthermore, relapses following the administration of vitamin B_6 have been difficult to explain. The indefinite distinction between acrodynia, generalized dermal symptoms and scaliness of the paws of experimental animals has likewise contributed a quota of confusion. Not only vitamin entities, but fats and certain fatty acids are involved in the problem (Burr and Burr, '29, '30; Birch and György, '36 and Birch, '38). Simplification of the basal diet and supplementation with pure entities in known amounts, as well as reduction in the multiplicity of source materials, have been of some value in facilitating an orderly integration of the relationships involved.

That the specificity of vitamin B_6 is dependent upon at least one other unknown factor, seems to have been well established

[1] Presented before the American Chemical Society, Division of Biological Chemistry, Cincinnati, Ohio, April 8–12, 1940.

by Lepkovsky et al. ('36), György and Eckardt ('39) and György et al. ('39). Liver and rice polish were the principal source materials used by these investigators. Bender and Supplee and co-workers (Bender and Supplee, '37; Ansbacher et al., '36; Supplee et al., '39) have also reported that acrodynia and dermal lesions in rats are prevented by a concentrate obtained from crude rice polish, and that this product supplemented with pure vitamin B_1 and pure riboflavin permits normal growth in rats, even after autoclaving at pH 8.5 for 5 hours at 120°C.

The availability of pure vitamin B_6[2] has permitted a systematic and comprehensive study of the interrelationship of this factor with other vitamin entities and concentrates of known properties. The rice polish concentrate to which reference has been made, when subjected to adsorption with fullers' earth, with or without previous removal of a substantial proportion of the protein and lipids, yields a filtrate fraction free from vitamin B_1, vitamin B_6 and riboflavin; this corroborates the observations of other investigators (Lepkovsky et al., '36; Edgar and Macrae, '37; and Oleson et al., '39). The present report contains data which interrelate the specificity of vitamin B_6 with a complementing and seemingly specific function of another factor contained in the treated rice polish concentrate (factor II according to Lepkovsky et al., '36) for the prevention of dermal lesions and promotion of normal growth of white rats. Nearly 1000 animals were involved in the studies reported.

EXPERIMENTAL

Since it is the policy of these laboratories to adhere to a standardized and proved basal ration with daily supplementation of pure entities or concentrates of proved characteristics, particularly for studies involving the entities of the vitamin B complex, this regimen was followed in the present work. The basal ration consisted of the following: vitamin free casein,[3]

[2] First made available to us through the courtesy of Dr. Lepkovsky and later through the courtesy of Merck and Company.

[3] Labco Brand, distributed by the Labco Products Department, The Borden Company, New York.

20 parts; sucrose, 69 parts; hydrogenated vegetable oil,[4] 3 parts; salt mixture no. 40,[5] 4 parts; powdered agar-agar, 2 parts; and cod liver oil (medicinal grade), 2 parts. This basal dietary was further supplemented with 12.5 γ pure vitamin B_1 and 10 γ pure riboflavin per rat per day, fed as a dosage in standardized solution. White rats 21 to 23 days old and weighing 45 to 50 gm. at weaning were placed in individual screen-bottom cages and supplied with the basal ration only, for a period of 1 week at which time the primary supplements and variables were introduced. The variants were graded amounts of crystalline vitamin B_6[6] and the rice polish derivative devoid of vitamin B_1, vitamin B_6 and riboflavin, which for convenience will be designated hereinafter as "rice polish factor II."

In order to determine whether or not this rice polish derivative was free of the factors mentioned, methods previously published (Supplee et al., '38, '39) were applied for the quantitative determination of vitamin B_1 and riboflavin. Test quantities as high as 2.11 gm. solids of the unautoclaved rice polish factor II failed to cure the polyneuritic symptoms in ten animals. When 150 mg. per rat per day were supplied as a potential source of vitamin B_1 for the growth requirement, the twelve test animals lost weight during the experimental period and all were dead within 6 weeks. The rate of growth of animals receiving test quantities of 150 mg. solids per rat per day as the sole source of riboflavin was substantially the same as that previously found for animals receiving a riboflavin-free diet, namely, an average of about 2 gm. per week during an 8-week period (Supplee et al., '39). The absence of vitamin B_6 was established by feeding test quantities up to 300 mg. solids per day as a potential source of this factor, while maintaining the primary supplements, vitamin B_1 and riboflavin. An incidence of 75 to 100% uncomplicated acrodynia developed in various groups of animals of from four to thirty-two individuals per group usually within a period of 6 to 8 weeks; weight gain was stimulated at a rate of about 8 to 10 gm. per

[4] Crisco.

[5] Steenbock, H., and Nelson, E. M., J. Biol. Chem., vol. 56, p. 355, 1923.

[6] Distributed by Merck & Co., Rahway, New Jersey.

week for about 3 weeks, remaining substantially constant or slowly declining thereafter. These data, obtained with the same basal diet by merely an interchange or withdrawal of a single primary supplement, substituting the unknown test material for the factor eliminated, yield evidence which is readily interpreted without the confusion incident to the introduction of variable basal components and unknown primary supplements. Therefore, it is concluded that the rice polish factor II used for the work described hereinafter was free of vitamin B_1, vitamin B_6 and riboflavin.

Since one of the primary objects of this work was to correlate the specificity of vitamin B_6 with another complementing factor under controlled conditions and with recognition of the quantitative relationships between the variants, it is appropriate to mention briefly at this juncture the role which certain fats and fatty acids are known to play in the prevention of dermal lesions associated with vitamin deficiency. The basal ration employed in these studies contained fats of known and substantially uniform characteristics. A high percentage incidence of dermatitis and typical acrodynia has been obtained in hundreds of negative controls receiving this basal diet during the past 5 years. These satisfactory results, even though subject to some variations in different groups of animals from time to time seemed to warrant its continuation for the present study, in lieu of a fat-free diet, or the introduction of a more restricted fatty acid component. Comparative studies have shown that no significantly greater incidence of acrodynia has resulted from this basal diet without the 3% hydrogenated vegetable oil;[7] the introduction of 10% delays the development of acrodynia to some extent, and 20% markedly reduces the percentage incidence. Ten per cent of corn oil, cottonseed oil[8] or olive oil substituted for the hydrogenated vegetable oil markedly reduced the dermal lesions and accentuated the curative response. Butterfat and coconut oil, and the fatty acids, stearic, palmitic, capric, caproic and myristic, in amounts from 0.5 to 5% of the basal ration are seemingly without effect;

[7] See footnote 4.

[8] Wesson oil.

whereas, lard (10%), oleic and linoleic acids delay and prevent the characteristic symptoms. No significant growth or differences in growth response resulted from the various fats or fatty acids. These comparisons which need not be further detailed in view of the more extended references in the literature have served to favor the 3% hydrogenated vegetable oil basal diet as one of choice for the comparative type of investigation with which this paper is primarily concerned.

By maintaining the primary supplements constant a comparative evaluation of pure vitamin B_6 and the rice polish factor II and combinations of these is readily obtained. The following data contain the composite record of 735 animals made up of comparable groups during a period of about 1 year. The rice polish factor II available as a water soluble concentrate containing approximately 80% total solids was diluted to 7.5–10% solids and autoclaved for 5 hours at 120°C. after pH adjustment to 8.5. The primary supplements and vitamin B_6 were pure products, the vitamin B_1 and vitamin B_6 being synthetic materials, whereas the riboflavin was a pure natural product isolated from milk. It will be observed (chart 1) that vitamin B_6 up to 10 γ per day in the absence of the autoclaved rice polish factor II stimulated growth for a period of only 2 to 3 weeks. The autoclaved rice polish factor II up to 100 mg. per day in the absence of vitamin B_6 stimulated growth to about the same degree. Neither substance in the absence of the other permitted normal development. However, when the two were combined, the vitamin B_6 being maintained at a constant level of 10 γ per day, and the rice polish factor supplied in graded amounts, the growth was commensurate with the amount of the latter supplied, 100 mg. per day being adequate for a normal rate of development of about 12 to 15 gm. per week.

Table 1 shows the percentage incidence of typical and nontypical acrodynia in the animals whose growth rates are plotted in chart 1. The designation "typical acrodynia" as used for diagnostic and record purposes throughout the course of this work refers to the edematous, exudative or florid dermal lesions with loss of fur on the paws, with or without accom-

panying involvement of the nose, mouth and ears. The "non-typical acrodynia" designation refers to the exfoliative dermatitis or dry scaliness of the paws; mild or insipid cases may show this condition to only a slight degree between the toes, whereas in more extreme cases it may affect the entire area of the paws. (Figures 1, 2 and 3 show gradations in severity of this condition; all such cases have been recorded as non-typical acrodynia.)

The character and severity of the dermatitis necessary for inclusion in the records as typical acrodynia required the

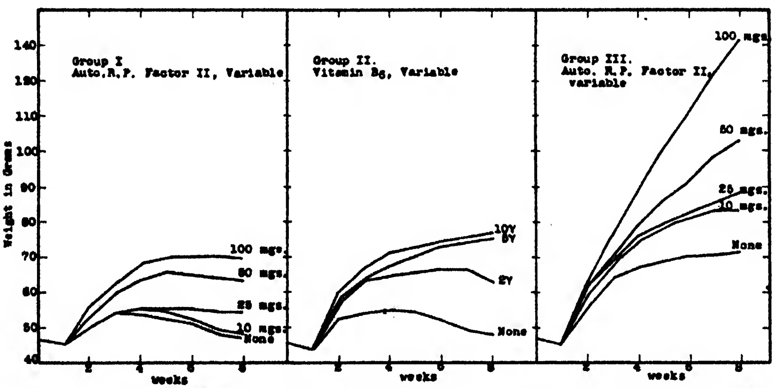

Chart 1 Growth of white rats as influenced by vitamin B_6 and unidentified vitamin factors in rice polish concentrate (factor II). (All animals received the basal diet only for the first week.) Groups 1 and II received 12.5 γ thiamin and 10 γ riboflavin only per day as primary supplements; group III received 10 γ vitamin B_6 daily in addition to the thiamin and riboflavin supplements.

manifestation of the florid lesions involving either the paws, or nose and mouth, or ears. While the groin and abdomen were occasionally affected, lesions in these areas were not numerous. (Figures 4, 5 and 6 show moderately severe or substantially average conditions of the paws, ears and mouth designated as typical acrodynia.) Neither generalized scaliness with exudative patches over the body nor any significant denuding has been noted during the course of these studies involving the regimen described. Ulcerated areas on the mucous lining of the lips, especially at the peripheral edges,

and on the tongue have been observed. Such cases have appeared most often on the regimen containing vitamin B_6 in the absence of the autoclaved rice polish factor II. Five milligrams of nicotinic acid and 200 γ of riboflavin daily for a period of 3 weeks failed to alleviate this condition; 100 mg. daily of

TABLE 1

Incidence of dermatitis (acrodynia) in white rats as influenced by vitamin B_6 and unidentified vitamin factors in rice polish concentrate (factor II)
(12.5 γ thiamin and 10 γ riboflavin per day as primary supplements)

FURTHER SUPPLEMENTS TO BASAL DIET	AMOUNT PER DAY	TYPICAL ACRODYNIA		NON-TYPICAL ACRODYNIA (SCALY PAWS)	
		Incidence	Av. time of appearance	Incidence	Av. time of appearance
		%	*weeks*	%	*weeks*
Autoclaved R.P. factor II	100 mg.	87	6.4	63	4.2
Autoclaved R.P. factor II	50 mg.	91	6.4	64	4.9
Autoclaved R.P. factor II	25 mg.	96	5.3	100	4.4
Autoclaved R.P. factor II	10 mg.	100	6.1	92	4.8
None	None	93	5.8	39	4.4
Vitamin B_6	10 γ	33	8.5	49	6.1
Vitamin B_6	5 γ	50	8.0	66	5.6
Vitamin B_6	2 γ	75	7.1	83	4.6
None	None	93	5.8	39	4.4
Vitamin B_6 + auto. R.P. factor II	10 γ 100 mg.	0	..	0	..
Vitamin B_6 + auto. R.P. factor II	10 γ 50 mg.	8	7.0	16	8.0
Vitamin B_6 + auto. R.P. factor II	10 γ 25 mg.	12	8.0	33	6.0
Vitamin B_6 + auto. R.P. factor II	10 γ 10 mg.	21	7.5	49	6.1
Vitamin B_6 + auto. R.P. factor II	10 γ None	33	8.5	49	6.1

the autoclaved rice polish factor II while continuing the vitamin B_6 stopped the necrosis and brought about complete healing in about 4 weeks. (Figure 7 illustrates the necrotic involvement at the end of the tongue.)

The non-typical lesions in all instances preceded the more severe or typical manifestations by about 1 week to 10 days on the average. Not all of the non-typical cases progressed to

the more severe type. The percentage of the mild cases which developed into the severe form was not substantially different among those groups receiving vitamin B_6 in the absence of the autoclaved rice polish factor II, than when the reverse scheme of supplementation was employed. However, a lower percentage incidence of both mild and severe cases occurred among the groups receiving vitamin B_6 in the absence of factor II, than was the case when the autoclaved rice polish factor II was supplied in the absence of vitamin B_6. When both substances were supplied the animals remained free of all dermal lesions if an adequate amount of factor II was provided. Detailed consideration of the records in the literature interpreted in conjunction with the present results leads to the conclusion that the classification of the lesions obtained during the studies here reported is a logical one and that the typical and non-typical conditions are merely degrees of severity resulting from inadequate amounts, or a disbalanced relationship between vitamin B_6 and another complementing factor or factors contained in the autoclaved rice polish factor II.

Earlier in this paper reference was made to the variability in percentage incidence of the dermal lesions in different groups of animals from time to time. During the course of attempts to determine the cause of this variation, the following experience was encountered: A given group of animals were developing characteristic symptoms between the fourth and the fifth week in accordance with expectations. Replenishment of the basal ration required purchase of a new lot of sucrose of a different brand than had been used previously. Coincident with the use of the basal ration made from the new supply of sugar there was a remission of the symptoms in many of the animals and progress of the disease was arrested in others. Inasmuch as there had been no change in the other components of the dietary these observations seemed to incriminate the sugar. In order to test the validity of this conclusion a quantity of commercial sugar was subjected to two recrystallizations, the purified sugar being used for compounding a new batch of basal ration, and the combined mother liquors and residual sugar used for another lot of basal ration. Eighty-eight ani-

mals were involved in a comparative study of the two sugars, the primary supplements and feeding regimen being maintained as previously described. It will be noted (table 2 and chart 2) that commercial sucrose may carry impurities to a degree which affects the percentage incidence of acrodynia

TABLE 2

Incidence of dermatitis (acrodynia) in white rats as influenced by the purity of the sucrose used in the basal ration

(12.5 γ thiamin and 10 γ riboflavin per day as primary supplements)

FURTHER SUPPLEMENTS TO BASAL DIET	AMOUNT PER DAY	INCIDENCE OF ACRODYNIA FROM RECRYSTALLIZED SUCROSE		INCIDENCE OF ACRODYNIA FROM MOTHER LIQUOR AND RESIDUAL SUCROSE	
		Typical	Non-typical	Typical	Non-typical
		%	%	%	%
Vitamin B_6	10 γ	60	90	10	50
Vitamin B_6	5 γ	75	100	25	70
Vitamin B_6	2 γ	75	100	75	100
Autoclaved R.P. factor II	300 mg.	75	..	25	..
Autoclaved R.P. factor II	100 mg.	90	..	50	..
Autoclaved R.P. factor II	50 mg.	100	..	75	..

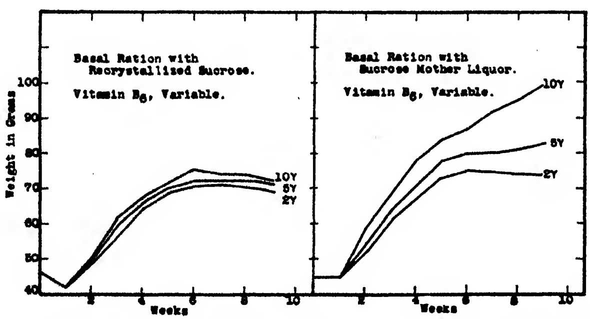

Chart 2 Growth of white rats as influenced by the purity of the sucrose used in the basal ration. (All animals received the basal diet only for the first week.) The animals in both groups received 12.5 γ thiamin, and 10 γ riboflavin and variable amounts of vitamin B_6 per day as primary supplements.

even under an otherwise carefully controlled dietary. This may conceivably account for the difficulties which certain laboratories experience in consistently developing the characteristic dermal lesions. György ('37) has reported that vita-

min B_6 is present in cane molasses, whereas beet molasses contains little or none of this factor. The offending lot of sugar encountered at this laboratory was a cane sugar.

Since there is a paucity of evidence in the literature concerning the reaction exhibited by mature animals deprived of factor II, it was deemed desirable to obtain data from such animals reared under controlled conditions. Young animals were raised to maturity on the basal ration and the supplements consisting of 12.5 γ vitamin B_1, 10 γ riboflavin, 10 γ vitamin B_6 and 150 mg. of the rice polish factor II per day for a period of 12 weeks. Two groups of twenty animals each with an equal number of males and females were used. One group received the rice polish factor II concentrate in raw, unautoclaved form, and the second group received the same material autoclaved for 5 hours at 120°C. following pH adjustment to 8.5. Following attainment in weight of about 180 gm. during the 12-week period the rice polish factor II was withdrawn, the other supplements being continued. The results from these comparisons (chart 3 and table 3) show that there was substantially no difference in rate of growth from the autoclaved and unautoclaved concentrate, all animals developing normally at a rate of 12 to 15 gm. per week. No pathological symptoms of any nature were observed. Following withdrawal of the rice polish factor II further increase in weight immediately ceased, substantially constant weight being maintained for a period of 20 weeks for those animals which survived this extended period. Survivals were greater for the group which had previously received the unautoclaved concentrate. Casualties in either of the groups were not preceded by loss of weight or emaciation; inanition and lack of vitality preceding final and unpredictable collapse constituted the characteristic reaction noted.

Acrodynia first occurred in the group which had previously received the autoclaved rice polish factor II 6 weeks following its withdrawal, whereas first occurrence did not appear until the twelfth week in the group which originally received the unautoclaved material. An increasing percentage incidence appeared in both groups with extended time. Since all animals

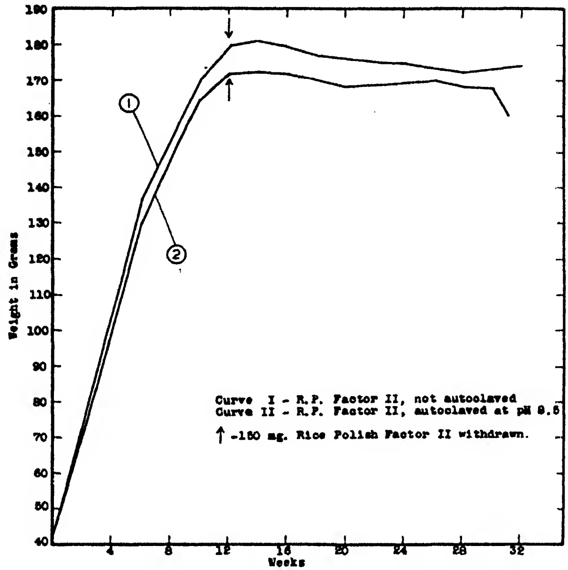

Chart 3 Development of white rats before and after elimination of rice polish factor II. All animals received 12.5 γ thiamin, 10 γ riboflavin, 10 γ vitamin B_6 and 150 mg. rice polish as daily supplements for 12 weeks.

TABLE 3

Percentage of survivors and incidence of acrodynia in mature animals following withdrawal of rice polish factor II (12.5 γ thiamin; 10 γ riboflavin; and 10 γ vitamin B_6 per day as primary supplements)

TIME AFTER WITHDRAWAL	SURVIVORS FOLLOWING WITHDRAWAL OF RICE POLISH FACTOR II		INCIDENCE OF ACRODYNIA FOLLOWING WITHDRAWAL OF RICE POLISH FACTOR II	
	Not autoclaved	Autoclaved	Not autoclaved	Autoclaved
weeks	%	%	%	%
6	100	100	0	10
8	100	100	0	15
10	100	100	0	15
12	100	85	10	35
14	85	45	25	35
16	75	30	25	40
18	55	25	40	50
20	30	20	40	55

received 10 γ vitamin B_6 per day throughout the entire observation period, these results are in confirmation of the evidence from young animals to the effect that the efficacy and specificity of vitamin B_6 for the prevention of characteristic dermal lesions are dependent upon a complementing factor or factors other than B_1 and riboflavin. This group of data as a whole also confirms and accentuates the evidence showing that other vitamin entities as yet unidentified are essential, not only for growth, but for prolongation of life of the mature animal.

SUMMARY

1. A standardized basal diet is presented which, when supplemented with a controlled daily intake of thiamin and riboflavin, permits the development of acrodynia in practically 100% of the cases usually within a period of 6 to 8 weeks.

2. Vitamin B_6 does not permit continued growth unless an unidentified factor (or factors) present in rice polish factor II is also supplied; likewise, this complementing substance does not permit growth in the absence of vitamin B_6. Appropriate amounts of both substances permit normal development in the presence of thiamin and riboflavin.

3. The specificity of vitamin B_6 for the prevention of acrodynia and exfoliative dermatitis of the extremities is dependent upon the complementing factor or factors contained in rice polish concentrate (factor II).

4. Impurities inherent in certain lots of commercial sucrose used as the carbohydrate for the basal ration may reduce the incidence and retard the development of acrodynia in experimental animals; such impurities may also stimulate a slight degree of growth when the basal ration is supplemented with controlled amounts of thiamin, riboflavin and vitamin B_6.

5. Autoclaving for 5 hours at 120°C. following pH adjustment to 8.5 does not destroy the anti-acrodynia and growth-promoting substance contained in the rice polish factor II.

6. Animals grown to maturity on the synthetic ration supplemented with requisite vitamin factors failed to gain weight following withdrawal of the rice polish factor II, but did maintain constant weight for several weeks; no significant loss of

weight, emaciation or other outward symptoms preceded casualties occurring during a 20-week period following withdrawal of this factor. Inanition and lack of vitality were the only characteristic signs noted just before the final collapse. Acrodynia occurred in a substantial percentage of the animals with an increasing incidence after an extended period.

Since the preparation of this manuscript a paper by Schneider et al. (J. Biol. Chem., vol. 132, p. 539, 1940) has appeared which presents data confirming significant observations and conclusions recorded herein.

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PLATE I

Explanation of Figures

1—Non-typical Acrodynia
(Severe Scaliness)

2—Non-typical Acrodynia
(Moderate Scaliness)

3—Non-typical Acrodynia
(Slight Scaliness)

4—Acrodynia
(Involvement of Mouth)

5—Acrodynia
(Involvement of Ears)

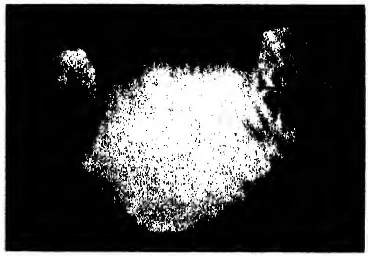
6—Acrodynia
(Involvement of Paws)

7—Acrodynia
(Necrosis at end of tongue)

1

4

2



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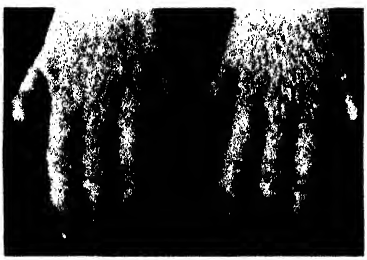

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CEREALS AND RICKETS

XII. THE EFFECT OF CALCIUM AND VITAMIN D ON THE AVAILABILITY OF PHOSPHORUS [1]

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Investigations designed to establish an optimal ratio of calcium to phosphorus for bone calcification have resulted in divergent conclusions.[2] As it has been established in recent years that most of the phosphorus of cereal grains is present as phytic acid, which is only partially available to the animal, and since different samples of a cereal grain may vary in the kind and amount of phosphorus present (Harris and Bunker, '35) it is plausible that there may be various optimal calcium-phosphorus ratios.

The production of rickets in rats on cereal rations has been associated with an unfavorable calcium to phosphorus ratio provided, of course, that a sufficiency of each for normal performance was present. With the use of cereal free rations low in phosphorus but complete in other respects (Schneider and Steenbock, '39; Jones, '39; Day and McCollum, '39) the complications arising from the use of cereals have become more evident. Recently Krieger, Bunkfeldt and Steenbock ('40 a) using a cereal free ration reported that the utilization of phytic acid phosphorus was markedly enhanced by the addition of vitamin D. Further investigations by the same

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] For references to most of the articles concerning calcium-phosphorus ratios see the paper by Lowe, Steenbock and Krieger ('39).

authors ('40 b) revealed that the calcium of calcium phytate was as readily available as that of calcium carbonate whether vitamin D was present or not. These results suggested the desirability of further work on the interrelations of calcium, phosphorus, and vitamin D with particular emphasis on determining the effect of calcium on the utilization of different forms of phosphorus. Since Zucker et al. ('22), Shelling ('25), and Shohl et al. ('28) have reported an acid-base factor in the production of rickets, due recognition was given to the possible effect of acid and basic calcium salts as well.

EXPERIMENTAL

The basal ration employed in these experiments was essentially the same as that used in previous investigations (Krieger, Bunkfeldt and Steenbock, '40 a, '40 b), with the exception that 1.33 parts of a salt mixture low in both calcium and phosphorus was substituted for the salts used before. The composition of this salt mixture was as follows: NaCl 105, KCl 120, $MgSO_4$ 90, $MnSO_4$ 0.20, $CuSO_4 \cdot 5H_2O$ 0.39, $K_2Al_2(SO_4)_4 \cdot 24H_2O$ 0.09, NaF 0.57, KI 0.05, and Fe citrate 16.15 parts respectively. The basal ration contained 0.04% phosphorus and 0.02% calcium as determined by analysis.

The calcium and phosphorus content of the diets was adjusted as already described, the phosphorus being kept at an optimal level by the addition of either phytic acid (Krieger, Bunkfeldt and Steenbock, '40 a) or inorganic phosphates. The phosphates were added as a neutral mixture of the anhydrous mono and di potassium salts. Calcium was added as precipitated calcium carbonate, anhydrous calcium chloride, or hydrated calcium sulfate (2 H_2O) to give approximate calcium-phosphorus ratios of 0.5/1, 1/1, 2/1, 4/1 and 6/1. One ration containing phytic acid phosphorus and another inorganic phosphorus, both without added calcium, were fed as negative controls. All rations were analyzed for total phosphorus (Fiske and Subbarow, '25), phytic acid phosphorus (McCance and Widdowson, '35), and calcium (Meloche et al., '33).

Viosterol, 45 U.S.P. units vitamin D per 100 gm. of ration, was used as the source of vitamin D.

The experimental conditions and criteria were the same as those used in previous investigations (Krieger, Bunkfeldt and Steenbock, '40 a, '40 b) except that calcium and phosphorus determinations on the blood sera were dispensed with. Paired feeding technique was used. The food intake was equalized

TABLE 1

Effect of calcium on phosphorus availability at calcium-phosphorus ratios of 1/1 or less

RATION NO.	Ca SOURCE	P SOURCE	Ca/ TOTAL P[1]	FOOD CONSUMED	CHANGE IN WEIGHT	WEIGHT OF BONE	WEIGHT OF ASH	BONE ASH
				gm.	gm.	gm.	gm.	%
1	None	Phytic acid	0.09	151	28.2	0.0931	0.0317	33.8
16	None	Inorganic	0.08	150	24.2	0.0947	0.0330	34.9
2	CO_3	Phytic acid	0.6	196	38.7	0.1194	0.0445	37.4
3	Cl_2	Phytic acid	0.6	195	38.0	0.1174	0.0442	37.4
4	SO_4	Phytic acid	0.6	199	43.1	0.1220	0.0461	37.7
20	CO_3	Inorganic	0.6	184	43.1	0.1146	0.0461	40.2
31	SO_4	Inorganic	0.5	176	49.5	0.1203	0.0469	39.0
5	CO_3	Phytic acid	1.0 [2]	221	55.0	0.1157	0.0443	38.2
6	Cl_2	Phytic acid	1.0 [2]	225	56.0	0.1373	0.0515	37.2
7	SO_4	Phytic acid	1.0 [2]	221	57.0	0.1249	0.0477	37.1
17	CO_3	Inorganic	1.0 [2]	216	54.3	0.1404	0.0611	43.4
29	Cl_2	Inorganic	0.9 [2]	209	59.4	0.1424	0.0607	42.6
32	SO_4	Inorganic	1.0 [2]	208	50.0	0.1328	0.0606	45.0

[1] The calcium phytic acid phosphorus ratio was practically identical.

[2] At these ratios three rats in one group died from an unknown cause after 3 weeks. Three rats were accordingly also taken for analysis at this time from the other groups leaving only three for the final analysis. The results from both were in harmony. All other analyses were based on six rats taken at 4 weeks.

between rats on identical calcium-phosphorus ratios after the first week. Each feeding experiment was terminated at the end of 4 weeks unless otherwise indicated.

RESULTS

It is evident from the data (tables 1 and 2) that a change in the level of calcium produced changes in growth, food consumption, weight of bone, weight of bone ash, and percentage of bone ash. In the negative control groups (table 1) there

were no differences in calcification regardless of the form of phosphorus given. It is quite apparent that here calcium was the limiting factor. When the calcium was increased to a calcium-phosphorus ratio of 0.5/1 there resulted a small but definite improvement in growth, food consumption, and calcification—but calcium was still the limiting factor. Slightly better calcification resulted with inorganic phosphorus than with phytic acid phosphorus although less ration was consumed.

TABLE 2

Effect of calcium on phosphorus availability at calcium-phosphorus ratios of 2/1, 4/1 and 6/1

RATION NO.	Ca SOURCE	P SOURCE	Ca/TOTAL P[1]	FOOD CONSUMED	CHANGE IN WEIGHT	WEIGHT OF BONE	WEIGHT OF ASH	BONE ASH
				gm.	*gm.*	*gm.*	*gm.*	%
8	CO_3	Phytic acid	1.9	237	64.3	0.1125	0.0328	29.1
9	Cl_2	Phytic acid	1.8	231	55.0	0.0991	0.0224	22.8
10	SO_4	Phytic acid	2.0	235	57.5	0.1103	0.0329	29.5
21	CO_3	Inorganic	1.9	214	53.2	0.1419	0.0636	44.8
30	Cl_2	Inorganic	1.7	217	59.2	0.1458	0.0673	45.8
33	SO_4	Inorganic	1.8	216	56.9	0.1488	0.0672	45.1
11	CO_3	Phytic acid	3.8	208	43.0	0.0944	0.0202	21.4
13	SO_4	Phytic acid	3.8	209	45.6	0.0975	0.0239	24.4
18	CO_3	Inorganic	4.3	215	51.6	0.1349	0.0569	42.0
34	SO_4	Inorganic	3.6	210	51.7	0.1368	0.0626	45.7
14	CO_3	Phytic acid	5.5	213	42.7	0.0964	0.0206	21.5
15	SO_4	Phytic acid	5.9	215	48.9	0.0985	0.0241	24.4
19	CO_3	Inorganic	5.7	218	52.8	0.1339	0.0553	41.1
35	SO_4	Inorganic	5.3	217	49.2	0.1351	0.0604	44.1

[1] The calcium phytic acid phosphorus ratio was practically identical.

When the calcium intake was increased to a calcium-phosphorus ratio of 1/1 no further increase in calcification resulted with phytic acid, although food consumption and growth continued to increase. However, with inorganic phosphorus better calcification resulted which was concomitant with better food consumption and better growth.

When the calcium content of the ration was increased still more to a calcium-phosphorus ratio of approximately 2/1 (table 2) a marked decrease in bone ash resulted with phytic

acid although there was little effect on food consumption or growth. On the other hand, inorganic phosphorus increased the percentage of bone ash slightly. Apparently calcium was no longer limiting calcification in the inorganic groups.

Further increases in the calcium content of the rations to calcium-phosphorus ratios of approximately 4/1 and 6/1 produced even more drastic changes than the 2/1 ratio. The availability of the phytic acid phosphorus was decreased still further but apparently reached the limit at the 4/1 ratio. These same levels, however, had little effect on the utilization of the inorganic phosphorus.

Variations in the source of the calcium produced small but persistent differences in the utilization of both forms of phosphorus, particularly at the higher ratios. Up to a calcium-phosphorus ratio of 1/1, where calcium obviously was the limiting factor, it was without effect (table 1). However, when the calcium was increased to a 2/1 ratio, the level being then slightly in excess of optimal (Krieger, Bunkfeldt and Steenbock, '40 b), the potentially acid salt, calcium chloride, produced a lower bone ash than the carbonate or the sulfate (table 2). This however was only true with phytic acid. With inorganic phosphorus no difference was observed. The carbonate gave the same result as the sulfate but when the calcium was increased to ratios of 4/1 and 6/1 the neutral salt, calcium sulfate, produced a slightly higher bone ash than the carbonate regardless of the source of the phosphorus. Calcium chloride was not used at these higher ratios, as some of the rats ate sparingly of these rations and failed to grow.

An acid-base effect has been observed before. Zucker et al. ('22) and Shelling ('25) reported an increased severity in rickets on acid diets and Shohl et al. ('28) reported good ash deposition with neutral diets, less with alkaline diets, and least with acid diets. On the other hand, McCollum et al. ('22) as well as Mellanby ('25) reported the acid-base factors as unimportant. Undoubtedly there are other factors such as solubility of the calcium salts which must be given due recognition.

The addition of vitamin D to rations of varying calcium-phosphorus ratios revealed differences in the utilization of phytic acid as contrasted with inorganic phosphorus (table 3). No difference was revealed when vitamin D was added to the negative control rations regardless of the source of the phosphorus. However, at the 2/1 ratios vitamin D markedly

TABLE 3

Effect of calcium and vitamin D[1] on phosphorus availability at various calcium-phosphorus ratios

RATION NO.	Ca SOURCE	P SOURCE	Ca/ TOTAL P[2]	FOOD CON-SUMED	CHANGE IN WEIGHT	WEIGHT OF BONE	WEIGHT OF ASH	BONE ASH (PLUS D)	BONE ASH (MINUS D)[3]
				gm.	gm.	gm.	gm.	%	%
1A	None	Phytic acid	0.09	152	16.3	0.1116	0.0393	35.3	33.8
16A	None	Inorganic	0.08	152	19.5	0.1063	0.0357	33.7	34.9
8A	CO_3	Phytic acid	1.9	235	57.1	0.1896	0.0924	48.3	29.1
9A	Cl_2	Phytic acid	1.8	235	48.0	0.1403	0.0629	44.7	22.8
10A	SO_4	Phytic acid	2.0	235	59.4	0.1733	0.0865	49.9	29.5
21A	CO_3	Inorganic	1.9	235	61.0	0.1973	0.1120	56.4	44.8
30A	Cl_2	Inorganic	1.7	235	64.9	0.2077	0.1137	54.8	45.8
33A	SO_4	Inorganic	1.8	235	65.7	0.2087	0.1313	54.9	45.1
11A	CO_3	Phytic acid	3.8	208	30.9	0.1218	0.0502	41.2	21.4
13A	SO_4	Phytic acid	3.8	208	36.8	0.1422	0.0659	46.5	24.4
18A	CO_3	Inorganic	4.3	208	44.6	0.1987	0.1065	53.5	42.0
34A	SO_4	Inorganic	3.6	208	47.9	0.2047	0.1134	55.3	45.7

[1] Forty-five U.S.P. units vitamin D (viosterol) per 100 gm.

[2] The calcium-phytic acid phosphorus ratio was practically identical.

[3] Values from identical rations minus vitamin D taken from tables 1 and 2.

improved the utilization of both forms of phosphorus, inorganic phosphorus producing the most bone ash but the phytic acid phosphorus giving the largest increment. It must be noted that the possible increment of improvement which could be achieved with inorganic phosphorus was definitely limited by the higher bone ash produced without vitamin D.

When the calcium-phosphorus ratio was increased to 4/1 the effect of vitamin D was still evident, but to only a slight degree with phytic acid. The increase in calcium caused a slight decrease in the percentage of bone ash of these latter groups over those on a lower ratio even though vitamin D was present. This effect was not observed with inorganic phosphorus. Apparently the calcium-phosphorus ratios are of no great importance when the phosphorus is present in the inorganic form.

Variations in the source of calcium again produced small but persistent differences in calcification even in the presence of vitamin D (table 3). At the 2/1 ratio calcium chloride produced less bone ash than the sulfate or the carbonate. At the 4/1 ratio the carbonate produced less bone ash than the sulfate, regardless of the source of phosphorus.

SUMMARY

In feeding experiments with rats the availability of phytic acid phosphorus was markedly affected by the calcium and vitamin D content of the diet. With an optimal intake of phosphorus and a calcium-phosphorus ratio of 1/1 phytic acid phosphorus was almost as readily utilized as the inorganic form. However, with an increase in the calcium-phosphorus ratio to 2/1 the availability of phytic acid phosphorus was markedly decreased. Further increases in the calcium content of the rations decreased the availability of phytic acid phosphorus still more. On the other hand, inorganic phosphorus was not markedly affected by an increase in calcium even up to a ratio of 6/1.

Different sources of calcium had a small but definite differential effect on the utilization of phosphorus. Calcium chloride produced a more severe rickets than either the carbonate or the sulfate. Calcium sulfate produced more bone ash than calcium carbonate.

Vitamin D improved the utilization of both forms of phosphorus. This effect was more pronounced with phytic acid since the increment of improvement possible within the limits of calcification was less with the inorganic form. Even in

the presence of vitamin D phytic acid phosphorus was not so readily available as the inorganic form. The aforementioned relative effects of different sources of calcium were also noted in the presence of vitamin D.

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THE BIOLOGICAL ASSAY OF RIBOFLAVIN [1]

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TWO FIGURES

In bioassays for riboflavin in which the growth of the rat is used, one of the most important problems is the choice of material to use as a source of the vitamin B-complex. This substance must be free of riboflavin so that the rats may be depleted of this vitamin, but adequate with respect to all other components of the vitamin B-complex. The object of the present study was to compare three such materials already widely used for this purpose as supplements to a purified diet, viz., extracts of rice polishings (Cook et al., '37; Day et al., '37; Helmer, '37; Supplee et al., '39), whole wheat (Bourquin and Sherman, '31) and yeast (Copping and Roscoe, '37). Having determined which of these was the most satisfactory for assays, it was our aim to set up criteria for depletion and to establish the relationship between graded doses and the gain in weight of rats depleted of riboflavin only. The final purpose of this study was to apply the information obtained to the performance of bioassays.

EXPERIMENTAL

Albino rats of the Yale strain, maintained on a satisfactory colony diet (The Vitamin Conference, U. S. P. XI, '31) were used. At the age of 16 to 19 days, when the young rats weighed

[1] A preliminary report was presented before the Pacific Division, American Association for the Advancement of Science at Stanford University, California, June 29, 1939.

between 30 and 40 gm., litters were transferred to a diet free of water-soluble vitamins which is described below. At 21 days the young were weaned and the mothers returned to the stock ration. The litters were transferred to individual cages having raised screen floors and continued on the basal diet plus daily doses of all the vitamins of the vitamin B-complex except riboflavin, administered in small dose cups. The rats were weighed three times weekly until depleted. Each experiment consisted of several groups of ten or more rats, distributed as uniformly as possible with regard to sex and litter. Every rat in a group received the same supplements.

The basal diet consisted of: corn starch,[2] 64%; casein,[3] 20%; hydrogenated cotton seed oil,[4] 10%; salt mixture,[5] 4%; cod liver oil,[6] 2%. This diet as well as water, U.S.P., was supplied ad libitum. Vitamins, other than riboflavin which was provided after depletion, and A and D which were included in the diet, were given separately throughout the depletion and assay periods. Vitamin B_1 was supplied as thiamin chloride,[7] 48 μg. daily. This large dose was considered necessary in order to minimize the variation of this factor, present in different quantities in the extracts under investigation, and to provide a sufficient amount so that vitamin B_1 would not be a limiting factor for growth even when large doses of flavin were given in an assay. The aggregate of thiamin given, approximately 24 international units per rat per day in all groups, approached the maximum dose for growth (Waterman and Ammerman, '35) and storage (Leong, '37). Solutions of thiamin were prepared according to the procedure in U. S. Pharmacopoeia XI, 1939 supplement.

The extracts used as sources of the water-soluble vitamins except riboflavin, were prepared as follows. Whole wheat

[2] Duryea's improved.

[3] Labco brand, vitamin-free.

[4] Crisco.

[5] Osborne and Mendel (no. 1 of U.S.P. XI).

[6] U. S. P.

[7] Merck.

extract was made according to the method of Bourquin and Sherman ('31) except that it was not dried on corn starch nor incorporated in the diet, but fed separately in daily doses so that it could be compared with other materials similarly administered. The aqueous alcoholic extract of 5 kg. of whole wheat was distilled under reduced pressure until the volume was 1 liter. The rice polishings concentrate [8] was the aqueous acetone extract described by Cook and Carroll ('36). This was used untreated or after one treatment with a small amount of fuller's earth. Each 100 gm. of rice polishings concentrate was diluted with 1.4 liters of phosphate-citric acid buffer at pH 4.2; 12 gm. of fuller's earth was added and the mixture was stirred for 15 minutes, filtered and evaporated in a partial vacuum at 50°C. For the yeast extract, two commercial yeast preparations were used after treatment with fuller's earth to remove riboflavin. Each 100 gm. of yeast extract was diluted with 2 liters of phosphate-citric acid buffer at pH 4.2; 30 gm. of fuller's earth was added. Sample A was treated with one lot of fuller's earth, sample B with two. Evaporation was carried out as for the rice polishings concentrate. The conditions chosen for adsorption, the amount of adsorbent, pH and time of treatment, were not those ordinarily used to effect the removal of either thiamin or vitamin B_6.

When rats had reached a plateau in their individual growth curves, they were considered depleted. All depleted animals fell into one of two categories, (a) those whose body weights were maintained for 1 week within 3 gm. and (b) those which had lost more than 3 gm. on 2 successive weighing days. The shortest depletion time allowed was 7 days after weaning. Some of the rats included in the assays were growing at a very slow rate, since a gain of 3 gm. in the week preceding an assay was permitted. Some of the negative control rats, continued for 4 weeks after depletion on the supplements without added riboflavin (rice polishings concentrate and thiamin only), grew slowly also, suggesting that the supplement used might have contained traces of riboflavin. How-

[8] 'Ryzamin-B' Burroughs Wellcome & Co. (U. S. A.) Inc.

ever, the prompt and sustained response to doses of riboflavin as small as 2.5 μg. daily shows clearly that the plateau was related to a deficiency of this vitamin. The question of the effect of arbitrary limits set for depletion time upon the results of experiments will be considered subsequently.

The criteria selected for evaluation of the extracts used as sources of the B-complex in riboflavin assays were (a) the time required for depletion and (b) the growth following the administration of a so-called "maximum dose" of pure riboflavin to rats depleted on the basal diet supplemented with the extracts under investigation. The first (a) should indicate whether the extract is free from riboflavin and second (b) should indicate whether the extract is adequate with respect to all other components of the vitamin B-complex.

The mean values for depletion time for rats on the supplements selected varied from 13 to 34 days (table 1). No significant differences [9] in mean depletion time were found when the value for the group receiving 150 mg. of "treated" rice polishings concentrate was compared with the values for all other groups, except the group receiving 150 mg. of yeast extract, sample A. In the latter case the time was significantly longer. The next group also, fed the same amount of another yeast extract had an even more extended depletion period. Using small groups of rats no significant shortening of the time required for depletion was evident when "treated" rice polishings concentrate was substituted for the "untreated" preparation (table 1). However, when larger numbers were used (table 1, last 3 assays) proof [10] that the treatment by adsorption significantly shortened the depletion time was adduced. Here the maximum time allowed for

[9] When the statistic t corresponded to a probability, P, equal to or less than 0.05, it was concluded that the mean of the group in question differed significantly from the mean of the group receiving 150 mg. of "treated" rice polishings concentrate. Fisher's tables ('38) were used.

[10] Here t corresponded to a value of P less than 0.01 when the mean of either group receiving "treated" rice polishings concentrate was compared with the mean of the group receiving the same amount of the untreated preparation.

depletion was 28 days when "treated" rice polishings concentrate was used, 56 days when "untreated" was used; some rats failing to show depletion within the time thus set were not used in the assay and not included in the figures quoted. About 25% were excluded because of the time limit when "untreated" rice polishings concentrate was used; less than 5% when the "treated" preparation was employed.

TABLE 1

Time required for depletion of riboflavin

SOURCE OF B-COMPLEX FACTORS [1]	NUMBER OF RATS	DEPLETION TIME REQUIRED (DAYS AFTER WEANING)	
		Mean	Standard deviation
150 mg. rice polishings concentrate, "treated"	13	16	± 8.5
150 mg. rice polishings concentrate, "untreated"	10	21	±10.3
75 mg. rice polishings concentrate, "untreated"	10	15	± 2.8
600 mg. extract of whole wheat	10	13	± 4.7
300 mg. extract of whole wheat	10	14	± 2.3
150 mg. yeast extract, sample A	10	29	± 8.2
150 mg. yeast extract, sample B	14 [2]	34	
75 mg. yeast extract, sample B	12	16	±11.0
150 mg. rice polishings concentrate, "treated"	59 [3]	13.1	± 2.5
150 mg. rice polishings concentrate, "treated"	60 [3]	17.0	± 8.9
150 mg. rice polishings concentrate, "untreated"	47 [4]	26.9	±14.0

[1] Each rat received crystalline thiamin in addition.

[2] Of this group, 29% were not depleted by the eighth week and were discarded. The mean includes these rats, assuming their depletion time to be 56 days, but the standard deviation was not determined.

[3] Rats were excluded from these assays when their depletion time was longer than 28 days.

[4] Rats were excluded fom this assay when their depletion time was longer than 56 days.

The second criterion used in judging the adequacy of the extracts employed as sources of the B-complex except riboflavin was the growth response, i.e., gain during a 4-week period after depletion when a "maximum dose" of riboflavin [11] was administered (fig. 1). This dose is defined as the smallest amount of riboflavin administered daily which will

[11] Labco brand. Solutions were prepared in sodium citrate buffer containing 25% alcohol at pH 5.0.

produce growth at a maximum rate in depleted rats; it has already been found (Edgar et al., '37) to be about 40 μg. When the five groups receiving 75 or 150 mg. of rice polishings concentrate, or 150 mg. of yeast extract (sample A and sample B) were compared, no significant difference[12] in gain during 4 weeks was found. The growth rate was significantly less than that of the above five groups when only 75 mg. of yeast extract was given, or when either of the two doses of wheat extract was used. The growth of rats given supplements which allowed the most rapid gains was compared with that occurring in our colony fed a mixed ration. The females

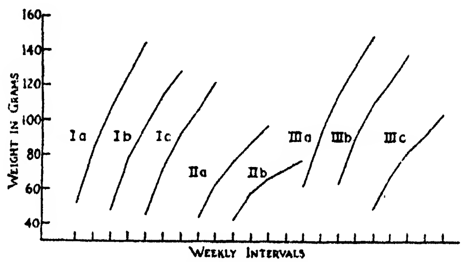

Fig. 1 Growth response in 4 weeks to a daily dose of 40 μg. of riboflavin. The sources of other B-complex factors were: I Rice polishings concentrate, *a* 150 mg., *b* 150 mg. "treated," *c* 75 mg.; II Wheat extract, *a* 600 mg., *b* 300 mg.; III "Treated" yeast extract, *a* 150 mg. sample A, *b* 150 mg. sample B, *c* 75 mg. sample B. Riboflavin was removed by adsorption from the samples labeled "treated."

in the experiment with 150 mg. of rice polishings concentrate were growing at a rate not significantly different from that of colony females for a corresponding 4-week period, whereas the males in the experiment were growing at a rate significantly less.

Since the responses of groups of rats to a 40 μg. dose of riboflavin, when the B-complex was provided by the wheat extract (either 300 or 600 mg. per day), was significantly less than that of all groups receiving rice polishings concentrate including the 75 mg. level, it was obvious that some B-complex

[12] The statistic t corresponded to a probability, P, greater than 0.05.

factor was limiting their growth. Under the conditions of our experiments, 600 mg. of the extract of wheat was not adequate for growth at a maximum rate. In the group receiving only 300 mg. of this extract, two rats developed florid dermatitis of the ears, like that usually ascribed to vitamin B_6 deficiency during the fourth week on the riboflavin dose. The swollen, scaly, inflamed condition of these ears disappeared in 2 weeks after changing the dose from 300 mg. of wheat extract to 75 mg. of rice polishings concentrate, the other supplements remaining the same. By this procedure the growth rate was doubled also. Evidence as to the presence of vitamin B_6 in curative amounts in the doses of rice polishing concentrate employed in our study is indicated by this experiment.

Although in equal doses yeast extract and rice polishings concentrate allowed approximately equal responses when a maximum dose of riboflavin was given (fig. 1), the fact that the groups given the yeast preparations (even after treatment with fuller's earth) had very long depletion times (table 1) was not overlooked when the final selection of the material most suitable for riboflavin assays was made.

Criticism might be made of the use of adsorption to remove riboflavin from rice polishings concentrate in view of the possibility that other essentials might be removed simultaneously even with the conditions carefully selected to avoid this possibility. Evidence that this is not the case is contributed as follows: (a) rapid growth, nearly equal to that of our colony, was obtained when only pure riboflavin was given in maximum dosage, (b) no symptoms appeared except those clearly due to riboflavin deficiency (described below) and (c) the administration of the vitamin most likely to be removed under our conditions of adsorption, namely vitamin B_6, to rats receiving all other vitamins including riboflavin, produced no difference in growth rate (table 2). Evidence as to the presence of adequate amounts of vitamin B_6 in the rice polishings concentrate employed in our study has been reported elsewhere (du Vigneaud et al., '39).

Having established the fact that rice polishings concentrate after one treatment by adsorption with fuller's earth was a satisfactory source of the vitamin B-complex components other than riboflavin in that it allowed a reasonably short depletion time when a dose was employed which would permit maximum growth, this material was used in an experiment in which the response of rats to graded doses of riboflavin was studied. The doses selected were 2.5, 5, 10, 20 and 40 μg. of riboflavin. There were ten rats in each group, five males and five females. Equations for regression lines relating the mean response, increase in weight in 4 weeks, to the logarithm of the dose, were calculated. The curves, drawn separately for males and females, seemed to be linear up to

TABLE 2

Results of experiment in which additional vitamin B_6 was used

SUPPLEMENTS	NUMBER OF RATS	GAIN IN WEIGHT, 4 WEEKS Mean	Standard deviation
		gm.	*gm.*
150 mg. "treated" rice polishings concentrate + 48 μg. thiamin + 10 μg. riboflavin	10	55.4	± 9.42
Same daily supplements + 200 μg. vitamin B_6[1] per week	10	56.0	±10.21

[1] Merck.

the 20 μg. dose, and proof for their linearity up to this dose level was adduced by methods outlined in Snedecor's text ('38). At dose levels higher than 20 μg., the values for both males and females indicate that the dose-response curves tend to flatten (compare Edgar et al., '37), although the point for the male response on the 40 μg. dose does not depart significantly from the linear relationship established by the calculated regression line.

Examination of the slopes of the regression lines for males and for females, up to the 20 μg. dose (fig. 2), shows that these are similar, the slope of the males being slightly steeper. The equations representing these lines are:

$$y = -2.64 + 71.24x \text{ (males)},$$
$$y = 1.64 + 68.66x \text{ (females)},$$

where y is the gain in weight in 4 weeks and x is the logarithm of the dose in micrograms. A comparison of the two slopes by means of a method described by Fisher ('38) shows that no significant difference in slope was established in this experiment ($P = 0.7$). Lindholm ('38) reports a considerably steeper slope for the regression line for the males than that for the females.

The only symptoms frequently observed in the nearly 500 rats used in these experiments were those of the eye. Only

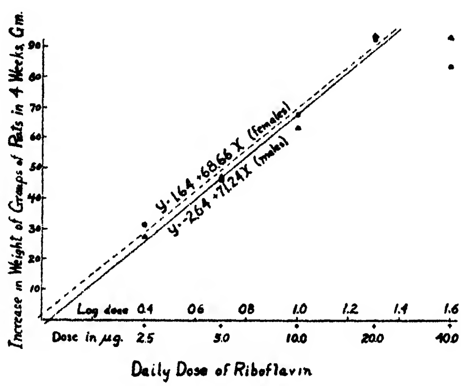

Fig. 2 Curves of response relating the logarithm of the dose to the increase in weight in 4 weeks: △ = mean response for males; ○ = mean response for females. The response to the 40 µg. dose (log of 40 = 1.6) was not used in calculating the equations given.

four cases of cataract were noted. Many rats, however, exhibited the blepharitis, alopecia of the lids, photophobia and lacrimation previously described (Day et al., '31; Bourne and Pyke, '35). The inflammation never appeared until after the third week of depletion, and was found most frequently in the negative controls although some rats receiving small doses of riboflavin also exhibited the symptoms. In one experiment where 130 rats were used, 30% of the negative controls, 24% of those receiving doses equivalent to 2.5 to 8 µg. and 5% of those receiving 10 µg. of riboflavin had inflamed eyes or eyelids. Since no microscopic study was

made, it is not certain whether vascularization of the cornea specific for riboflavin deficiency (Bessey and Wolbach, '39) developed.

A number of rats showing eye symptoms were treated with large doses of a "filtrate factor" concentrate or vitamin A (cod liver oil) to determine whether the condition was curable by these factors. Cures were not effected. However, when a dose of 40 μg. of riboflavin was given daily, improvement began immediately, and in every case cures were complete in about 2 weeks. El-Sadr ('39) described similar cures with pure riboflavin in rats receiving liver or yeast preparations as sources of the B-complex. The occurrence of specific riboflavin deficiency symptoms and their cure under our experimental conditions provides justification for the use of the non-specific growth method for assay purposes.

Using the method outlined in this paper, riboflavin containing materials, principally yeast concentrates, have been assayed. Throughout the assays the basal diet supplemented daily with 150 mg. of "treated" rice polishings concentrate and 48 μg. of thiamin was given to rats of weight and age specified earlier in this paper. When the graphic method was used in interpreting results two control groups were given doses of riboflavin of such size that the mean responses lay on the linear portion of the dose-response curve. An attempt was made to select a dose of the "unknown" so that the response would fall between the responses of the two control groups. When an algebraic method was employed to interpret the results by calculation of the regression lines, two or three groups for both control and "unknown" were included in the assay, the doses being in the ratio of 1:2 or 1:2:4. In several assays set up in this way, each group comprising ten rats, the limits of error within which the potency could be expected to fall in twenty-one trials out of twenty-two, were found to be approximately 79 and 127%. By setting a limit on the time for depletion, i.e., excluding rats not depleted in 28 days, the accuracy of the assays was increased somewhat. In a group of assays without the time

limitation, the average standard deviation for gain in weight in 4 weeks was 15.9 gm.; in another group of assays with this limitation, the average standard deviation was 9.5 gm. Other devices for decreasing the limits of error of the assay such as increasing the number of rats per group, or the number of groups may be employed.

The authors gratefully acknowledge the generous assistance of Dr. Edwin J. deBeer of our staff in the preparation of this manuscript, especially the statistical part.

SUMMARY

A study of the rat growth method for the biological assay of riboflavin has been made. Comparisons of extracts of rice polishings, whole wheat and yeast, used as sources of the vitamin B-complex other than riboflavin in these assays, indicated that the rice polishings concentrate after one adsorption with fuller's earth is the most satisfactory supplement. Under the experimental conditions employed, neither the wheat extract nor the yeast extract satisfied the two requirements as well as did rice polishings concentrate, namely, freedom from riboflavin and adequacy with respect to all other components of the vitamin B-complex. It was established that the relationship between the logarithm of the dose and the response, gain in weight in 4 weeks, is linear up to the 20 µg. dose and that the regression lines for males and females are practically identical. Methods whereby the results thus described may be utilized in the estimation of the riboflavin content of unknowns and the limits of error of such assays are suggested.

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THE EFFECTS PRODUCED BY DECREASING THE CALCIUM AND PHOSPHORUS INTAKE ON CALCIUM AND PHOSPHORUS ABSORPTION AND DEPOSITION AND ON VARIOUS BODILY CONSTITUENTS OF THE RAT

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An excess of calcium and phosphorus in the diet of albino rats was found in a previous study (Haldi, Bachmann, Wynn and Ensor, '39) to result in a significant diminution of the fat content of the body, an increase in the percentage water and the renal lesions that had been previously described by MacKay and Oliver ('35) and attributed by them to the high intake of phosphate. The present investigation was undertaken to determine the effects of lowering the calcium and phosphorus intake on the calcium and phosphorus balance and on the same bodily constituents as in the previous study. In addition the composition of the bone was also studied.

PROCEDURE

The general procedure of these experiments was the same as in the former investigation (Haldi et al., '39). The animals were selected at weaning in groups of three of the same sex from the same litter and were approximately the same weight. They were fed the Wistar diet for 1 week, each animal receiving the same amount of food as its litter mate. They were then placed on the experimental rations. The basal or control ration, which will be designated as ration 1, consisted of 72% sucrose, 18% casein, 6% yeast and 4% of the

Osborne-Mendel salt mixture ('19), which was slightly modified by reducing the calcium carbonate and phosphoric acid content so that the ration contained 1% calcium and 0.57% phosphorus. The other two rations, to be referred to as rations 2 and 3, were of the same composition except for the salt mixture which was changed with respect to calcium and phosphorus so that rations 2 and 3 contained approximately 67% and 40%, respectively, as much calcium and phosphorus as ration 1. The average calcium content of the three rations was, respectively, 1.0, 0.67, and 0.40% of the food mixture and the phosphorus content 0.57, 0.39, and 0.24%. The amount of calcium and phosphorus present in the mixture was determined by analysis. The Ca/P ratio in the three rations ranged from 1.7 to 1.8. Eight males and the same number of females were placed on each of the three rations. A 6:1 mixture of cod liver oil and linoleic acid was administered daily to each animal by medicine dropper. The daily intake of vitamin A was approximately 1350, and of vitamin D, 25 international units.

The food intake of the three litter mates in each group was maintained as nearly equal as possible from day to day and completely equalized once a week. The caloric value of the total food intake of litter mates on the three rations was therefore the same at the conclusion of the experiment, although there was a slight difference in the bulk of the food mixture that was ingested because of the difference in the salt content. The average total amount of food consumed, exclusive of the salt mixture, was 847 gm. for the males and 660 gm. for the females. The feces were collected throughout the experiment and analyzed for calcium and phosphorus.

When the animals had been on the experimental ration for 70 days, they were fasted for 24 hours with free access to water, then decapitated, ground to a homogeneous mass and analyzed for water, fat, protein, calcium, and phosphorus in the manner described elsewhere (Haldi, Bachmann, Wynn and Ensor, '39). Before the bodies were ground, the right femur was removed and weighed and subsequently dried in

an oven at 110°C. The bone was then freed from organic material by the ashing method of Gabriel as modified by Morgulis ('31), dissolved in HCl and analyzed for calcium and phosphorus.

RESULTS

In order to facilitate a comparison of the results of these experiments with those obtained with a larger calcium and phosphorus intake, the data are presented in the same tabular form as in the earlier report. The probability of the differences between averages being due to chance, as shown in table 3 for the various body constituents and designated in the text as P, was determined by applying the t test of Fisher ('32) using the formula for t which takes into account the correlation between litter mates.

Calcium and phosphorus absorption. The difference between the amounts of calcium and phosphorus ingested and those recovered in the feces will be referred to for descriptive purposes as the "amount absorbed." The objective of these experiments makes it unnecessary to take into account the excretion by the intestinal wall.

The data in table 1 show that with a progressive decrease in the calcium and phosphorus intake there was a progressive decrease in absorption. Although absorption and deposition diminished with the smaller intake, part of the ingested calcium and phosphorus was excreted in the feces. The average fecal calcium values on the three rations proved to be 66%, 57% and 34%, respectively, of the calcium intake. The phosphorus in the feces was, respectively, 55%, 46% and 33% of the amount ingested. It is impossible to state whether the higher percentages in the case of calcium excretion are to be accounted for by a smaller absorption of calcium than of phosphorus or by a larger excretion of calcium through the intestinal wall.

Absorption of food material. The organic matter in the feces of the males and females, as determined by subtracting the total ash from the dry weight, was 26, 23 and 20 gm.,

respectively, on the three rations. With the decrease in the calcium and phosphorus content of the diet, there was therefore a slight increase in the amount of food material absorbed. The difference, however, of 6 gm. in absorption on diets 1 and 3 was probably inconsequential as it was less than 1% of the total organic material ingested throughout the experiment.

Urinary excretion of calcium and phosphorus. Direct determination of the urinary excretion of calcium and phosphorus by analysis of the urine collected from day to day was impracticable under the conditions of the experiment. The food mixture was kept in conical cups to safeguard against spilling, but a small amount would adhere to the front feet which were almost invariably inserted into the cups when the animals were feeding. Although the amount of the food mixture lost in this way was inconsequential it was thought that it would be sufficient to vitiate the results of urinary analysis since the amount of calcium and phosphorus excreted daily in the urine was extremely small.

It is possible, however, to determine approximately the quantity of calcium and phosphorus excreted in the urine by subtracting the amount deposited in the body from the amount absorbed. The quantity absorbed will be found in table 1. The amount deposited was obtained by subtracting 0.28 gm. from the total calcium in the body at the conclusion of the experiment and 0.18 gm. phosphorus from the total phosphorus as shown in table 2. These subtractions were made to allow for the amounts of these elements in the bodies of the animals at the beginning of the experiment. The values 0.28 and 0.18 gm. were obtained from analysis of animals at the age when the experimental feeding was begun. These rats were of the same weight and maintained under the same conditions as the experimental animals.

The calculations showed that the average calcium excretion in the urine over 70 days for the entire group of males and females was 0.46, 0.23 and 0.20 gm., respectively, on rations 1, 2 and 3 and the phosphorus excretion 0.79, 0.47 and 0.16 gm. These data are presented to show that with a decrease in the

TABLE 1

Calcium and phosphorus absorption over a period of 70 days with different calcium and phosphorus intake[1]

RATION[2]	CALCIUM INTAKE	CALCIUM IN FECES	AMOUNT ABSORBED[3]	DECREASE IN ABSORPTION COMPARED WITH CONTROLS	PHOSPHORUS INTAKE	PHOSPHORUS IN FECES	AMOUNT ABSORBED[3]	DECREASE IN ABSORPTION COMPARED WITH CONTROLS
Males								
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	8.82	5.77	3.05	—	5.05	2.72	2.33	—
2	5.84	3.37	2.47	0.58	3.42	1.58	1.84	0.49
3	3.46	1.18	2.28	0.77	2.06	0.65	1.41	0.92
Females								
1	6.87	4.50	2.37	—	3.93	2.19	1.74	—
2	4.56	2.50	2.06	0.31	2.68	1.23	1.45	0.29
3	2.72	0.89	1.83	0.54	1.61	0.54	1.07	0.67
Males and females								
1	7.84	5.14	2.70	—	4.49	2.46	2.03	—
2	5.20	2.94	2.26	0.44	3.05	1.41	1.64	0.39
3	3.09	1.04	2.05	0.65	1.84	0.60	1.24	0.79

[1] There were eight males and eight females on each ration; the values in the table are averages.

[2] Ration no. 1 contained the Osborne-Mendel salt mixture with slight modifications (see text); no. 2, approximately 67%, and no. 3, 41% as much calcium and phosphorus as ration no. 1.

[3] The difference between the intake and the amount recovered in the feces is regarded for convenience as the "amount absorbed."

TABLE 2

Composition of rats with different calcium and phosphorus intake[1]

RATION	INITIAL WEIGHT	FINAL WEIGHT AFTER 24-HOUR FAST	INCREASE OVER INITIAL WEIGHT	WATER CONTENT	DRY WEIGHT	FAT	PROTEIN (N × 6.25)	CALCIUM	PHOSPHORUS	Ca/P RATIO
Males										
	gm.	*gm.*	*%*	*%*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
1	34	259	662	57.8	109.3	44.6	47.8	2.72	1.56	1.7
2	33	254	670	56.7	110.0	48.3	44.8	2.50	1.48	1.7
3	34	257	656	57.8	108.5	46.7	44.8	2.32	1.42	1.6
Females										
1	33	179	442	58.9	73.6	28.1	32.7	2.31	1.28	1.8
2	34	180	429	58.6	74.5	29.5	31.5	2.12	1.21	1.8
3	34	172	406	60.9	67.3	25.1	30.2	1.94	1.10	1.8

[1] Each value in the table is an average derived from eight experiments.

calcium and phosphorus intake there was a diminution in their output in the urine. It is worthy of note that a positive calcium and phosphorus balance occurred with the smallest intake.

Water intake. The averages of the water intake of the males and females on the three rations for the entire experiment were, respectively, 1160, 1163 and 1146 cc. It is of interest to note in this connection that on a diet containing approximately only 0.015% phosphorus, Day and McCollum (cited by McCollum, Orent-Keiles and Day, '39) found no marked increase in water consumption as is said to occur in phosphorus deficient animals.

Body weight and body analysis. The percentage gains in body weight and the absolute dry weights of the males were not significantly different on the three rations (table 2). The females, however, showed a significantly lower percentage gain on ration 3 than on ration 1. The dry weight of the females was also definitely smaller for the animals on ration 3 than for those on rations 1 and 2, whereas the dry weights of the males were the same on the three rations. While the percentage gains in weight and the dry substance of the bodies were smaller in the females on ration 3, the percentage water content was 2.0% and 2.3% higher than on rations 1 and 2, respectively. These higher percentages were definitely significant as P was equal to 0.013 and 0.005, respectively. The percentage water content in the males was not significantly different on the three rations.

The fat contents of the males were not significantly different on the three rations. The bodies of the females, however, contained a definitely smaller amount of fat on ration 3; but the difference on rations 1 and 2 was not significant.

The deposition of body protein in both the males and females was adversely affected by reducing the calcium and phosphorus intake. In the males the protein in the body was the same on rations 2 and 3 but in each instance definitely less than on ration 1. In the females the protein deposited was not significantly reduced on ration 2, but as shown in table 3, it was definitely less on ration 3.

TABLE 3

Statistical analysis

	CALCIUM		PHOSPHORUS		DRY WEIGHT		FAT		PROTEIN	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Comparison of body constituents on rations 1 and 2										
Mean difference (gm.)[1]	+0.22	+0.19	+0.08	+0.07	−0.7	−0.9	−3.7	−1.4	+3.0	+1.2
Standard deviation	±0.22	±0.17	±0.13	±0.12	±7.0	±4.0	±6.7	±5.2	±1.3	±1.96
Probability	0.016	0.012	0.072	0.094	0.233	0.30	0.09	0.253	<0.001	0.075
Comparison of body constituents on rations 1 and 3										
Mean difference (gm.)[1]	+0.40	+0.37	+0.14	+0.18	+0.8	+6.3	−2.1	+3.0	+3.0	+2.5
Standard deviation	±0.22	±0.25	±0.10	±0.11	±6.8	±2.9	±6.6	±3.1	±3.1	±2.1
Probability	<0.001	0.004	0.003	0.002	0.33	<0.001	0.214	0.018	0.019	0.008
Comparison of body constituents on rations 2 and 3										
Mean difference (gm.)[2]	+0.18	+0.18	+0.06	+0.11	+1.5	+6.8	+1.6	+4.4	0	+1.3
Standard deviation	±0.12	±0.15	±0.08	±0.05	±7.1	±5.1	±5.6	±3.1	—	±1.6
Probability	0.003	0.018	0.041	<0.001	0.31	0.004	0.238	0.009	—	0.034

[1] + indicates a greater average content on ration 1; — the reverse.

[2] + indicates a greater average content on ration 2; — the reverse.

Correlatively with the decrease in absorption of calcium and phosphorus on rations 2 and 3 there was a smaller deposition of these elements in the body. The differences in the calcium content of the body on the various rations were all significant as shown in table 3. The calcium content of the bodies on the three rations was, respectively, 1.05, 0.98 and 0.90% of the body weight in the males and 1.29, 1.18 and 1.13% in the females. The percentage calcium in our control group was slightly higher than the normal values of 0.96% for males and 1.16% for females 90 days of age, as reported by Whitcher, Booher and Sherman ('36). The diet of their animals consisted of one-third whole milk powder, two-thirds ground wheat, and contained 0.83% calcium and 0.43% phosphorus. It will be observed that the calcium and phosphorus content was nearly the same as in our control ration. Our animals were 100 days old when sacrificed and heavier than those of Sherman and his co-workers. The difference in age and weight doubtless accounts for the difference in percentage calcium, because our values were approximately the same as for their older animals which were nearly equal in weight to our 90-day-old rats.

The phosphorus in the bodies of the animals on the three rations was, respectively, 0.60, 0.58 and 0.55% of the body weight for the males and 0.71, 0.67 and 0.64% for the females. Although the differences in the phosphorus content of the body were small, the differences between the animals on ration 3 as compared with those on rations 1 and 2 were significant. The difference, however, between rations 1 and 2 was not significant.

Bone. As shown in table 4, a progressive decrease in the calcium and phosphorus intake produced no change in the organic material of the femur but was accompanied by a progressive decline in the wet weight, dry weight, ash, calcium, and phosphorus content. Since the same order of differences was observed in the bones of the males and females on the different rations, the data on the two sexes were combined for statistical analysis. The lower values on ration 2 as com-

pared with those on ration 1 and on 3 as compared with ration 2 were definitely significant with one exception. The difference in the wet weight with respect to rations 2 and 3 was not significant as P was equal to 0.14. The lack of significance of the difference between these groups was doubtless due to the irregularity that occurred in the males, namely, a slightly heavier weight of the bone when on ration 3 as compared with 2. The difference of 26 mg. in the weight of the femur of the females on these two rations was definitely significant (P = 0.02).

TABLE 4

Femur—weight and analytical data

RATION	WET WEIGHT	DRY WEIGHT	TOTAL ASH	ORGANIC MATTER	TOTAL CALCIUM	TOTAL PHOSPHORUS	Ca/P RATIO
				Males			
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
1	716	453	309	144	105.7	46.1	2.3
2	689	437	298	139	100.7	44.1	2.3
3	697	421	275	146	93.9	41.1	2.3
				Females			
1	576	379	266	113	89.2	38.4	2.3
2	570	375	255	120	86.3	37.2	2.3
3	544	324	212	113	71.5	30.8	2.3
				Males and females			
1	646	416	288	129	97.5	42.3	2.3
2	630	406	277	130	93.5	40.7	2.3
3	621	373	244	130	82.7	36.0	2.3

The values for calcium in the bone relative to the three rations and given in table 4 represented, respectively, 23.3, 23.0 and 22.3% of the dry weight of the bone in the males and 23.5, 23.0 and 22.1% in the females; those for phosphorus were 10.2, 10.1 and 9.8% in the males and 10.1, 9.9 and 9.5% in the females. The reduction of the calcium and phosphorus in the case of ration 2 as compared with ration 1 was extremely small, but it is worthy of note that the same results were obtained in the males and females. The total calcium and phosphorus in the bone in the case of ration 2 was 96% and

with ration 3, 85% of the amount present when the control ration was fed.

Although the total organic matter in the bone was not significantly different on the three rations, the percentage organic matter with reference to the dry weight showed a progressive increase with a reduction of the percentage calcium and phosphorus in the food mixture. On rations 1, 2 and 3 it was, respectively, for the entire group of males and females 31.0, 32.0 and 34.9%. The difference between rations 1 and 2 was probably significant ($P = 0.042$); the difference between rations 1 and 3 was definitely significant ($P = 0.0001$).

DISCUSSION

The reduction in the weight of the bone that resulted from the smaller intake of calcium and phosphorus, and the change that occurred in the composition of the bone, namely, a lower percentage of calcium and phosphorus together with a higher percentage of organic material, appears to have been significant. It is possible that such a change weakened the bones. This question, however, should be put to an experimental test before definite conclusions are formulated.

While a lowering of the calcium-phosphorus intake in these experiments led to a decrease in the calcium, phosphorus, and protein deposition in the body, no conclusions can be drawn at the present time with regard to their practical import. Further investigation will be required to determine whether longevity, reproductivity, spontaneous activity, resistance to fatigue, endurance and the various physiological processes of the organism are adversely affected by the diminution in the calcium, phosphorus, and protein that occurred. No differences in the general condition of the animals on the different rations could be detected from gross observation.

A slight impairment in digestibility or absorption with the higher calcium and phosphorus intake is indicated by the larger amount of organic material in the feces. Since the animals did not gain more weight on a larger calcium and phosphorus intake notwithstanding a slightly greater

absorption of food material, it would appear that a slightly greater waste of energy as heat occurred in these animals than in those on the higher calcium and phosphorus intake.

In these experiments it was deemed advisable to maintain the Ca/P ratio constant; consequently, it is impossible to state whether the results obtained are to be attributed to the deficiency in the intake of calcium or of phosphorus or of both. To establish this point further work is needed in which the percentage calcium in the food mixture is reduced while the percentage phosphorus is maintained constant and vice versa, the normal ratio being disregarded.

SUMMARY

The effects of lowering the calcium and phosphorus intake of albino rats were studied with respect to the calcium and phosphorus absorption over a period of 70 days and the various constituents of the body and the femur.

The basal ration contained 1% calcium and 0.57% phosphorus. Ration 2 contained approximately two-thirds and ration 3 less than one-half the amount of calcium and phosphorus in the basal ration. The Ca/P ratio was the same in the three rations.

On the lower calcium and phosphorus rations there was a progressive decrease in the amount of these elements in the feces. An appreciable amount of both calcium and phosphorus was nevertheless recovered on the ration giving the lowest intake.

Calculations indicate that over a period of 70 days on the three rations, 0.41, 0.23 and 0.20 gm. calcium and 0.79, 0.47 and 0.16 gm. phosphorus were excreted in the urine.

A decrease in the calcium and phosphorus content of the diet was accompanied by a slight increase in the amount of food material absorbed.

There was no significant difference in the water intake on the three rations. The animals were allowed to drink ad libitum.

The progressive decrease in the calcium and phosphorus percentage of the food mixture resulted in a corresponding diminution in the wet weight, dry weight, ash, calcium and phosphorus content of the bone and an increase in the percentage of organic material.

The calcium and phosphorus content of the body was correlatively less with the lower calcium and phosphorus intake.

Reduction in the calcium and phosphorus intakes had no effect on the body weight, dry weight or fat deposition except in the females on ration 3. In this case the gain in weight, the dry weight and fat content of the body were less than on rations 1 and 2.

The protein content of the body was less with the low calcium and phosphorus intake than on the basal ration.

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THE RELATION OF PHYTIN TO THE CALCIFYING ACTION OF CITRATES [1]

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For several years citric acid and its salts have been occasionally recognized as possibly beneficial in the prevention of rickets in infants (Weissenberg, '28; Siwe, '38). Shohl ('37), Hamilton and Dewar ('37) and Hathaway and Meyer ('39), using the Steenbock-Black and McCollum ricketogenic diets, demonstrated that citrates have both a curative and preventive effect in rats when fed at high levels. A mixture of the free acid and the sodium or potassium salt was more antiricketic than either the acid or salts alone (Shohl, '37; Hathaway and Meyer, '39). That the beneficial effects are not attributable to acid-base factors is indicated by Shohl ('37) who found that among the organic acids tested only citrates and tartrates were effective. Hamilton and Dewar ('37), recognizing that citrates (and tartrates) form complexes with calcium, suggested that the antiricketic effect is possibly due to a decrease in available calcium, thus sparing phosphorus which is in relatively low concentration in the diets used. There is ample evidence that calcium forms a soluble complex with citrates in which the calcium ceases to be positively ionized (Starkenstein, '14; Shelling and Maslow, '28; Shear, Kramer and Resnikoff, '29; Greenberg and Greenberg, '32-33; Hastings et al., '34). Shohl and Butler ('39) have reported two cases of human rickets which they believe were benefited

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by citrate administration. Eliot and Park ('37) concluded that, "Perhaps by means of sodium citrate it will be possible greatly to accelerate the cure of rickets and to treat successfully the forms of the disease which are refractory to all but enormous doses of vitamin D." Since rickets is characterized by inadequate mineralization of growing bone (Shohl, '39, p. 147) the relation of citrates to bone mineralization, in general, and rickets prevention and cure, in particular, should be further investigated.

Because the experimental diets used by previous investigators contained a large percentage of corn, known to contain variable amounts of biologically unavailable phosphorus (Harris and Bunker, '35), it seemed important to determine the calcifying effect of citrates on bones, using a purified diet containing different amounts of calcium and phosphorus of high biological availability. In case the beneficial effect on ossification could not be produced with purified diets, but only with corn diets containing an excess of calcium carbonate, it would seem possible that the effect might be related to the phytin of the unpurified diets. Lowe and Steenbock ('36a) found that, "Phytin-P is not completely unavailable to the rat, and secondly that the small amount which might be available is rendered almost completely unavailable by the presence of $CaCO_3$." It would be a plausible assumption, therefore, that citrates added to diets containing phytin and an excess of calcium carbonate reduce the ricketogenic (anticalcifying) action of such diets by forming a citrate complex with the calcium, thus permitting more of the phytin phosphorus to be hydrolyzed and utilized.

EXPERIMENTAL

Young rats weighing 45 to 60 gm. were used. The sexes and litters were equally distributed in each experiment. Three or four rats were kept in each cage. Food was given ad libitum.

At the end of each experiment, 20 or 21 days, the animals were killed with ether and the left hind leg of each was removed. Soft tissue was loosened by boiling 3 minutes in

distilled water. The bones were carefully cleaned and the femur, tibia and fibula of each leg were extracted 48 hours with ethyl alcohol and 48 hours with ethyl ether. After drying 24 hours at 102°C., they were placed in a cold muffle furnace and ashed 14 to 18 hours at about 550°C. Bone ash (total ash and percent ash) determination was relied upon to appraise the calcifying effectiveness of citrates in the various diets because it is entirely objective, and in a procedure as was employed here, the method is more accurate than the alternative histological or roentgenological technics (Coward, '38). Lowe and Steenbock ('36b) depended exclusively upon bone analysis (total ash and percent ash) in studies on the ricketogenic properties of corn. Such a procedure is essential if the antiricketic effectiveness of a substance is to be measured in terms with practical meaning, i.e, the degree to which mineralization of bone is facilitated.

The composition of all basal diets used is given in table 1.

TABLE 1

Basal diets

CONSTITUENTS	1	2	3	4	5	6	7	8
	%	%	%	%	%	%	%	%
Yellow corn	73	73	73					
Wheat gluten	20	20	20					
NaCl	1	1	1					
Casein, acid washed				15	15	15	15	15
Edestin				3	3	3	3	3
Dextrin				20	20	20	20	20
Sucrose	3	2.5	2	40.7	40.4	39.9	39.2	39.4
Yeast, bakers'				5	5	5	5	5
Salts no. 4 (Ca-P free)				3	3	3	3	3
Hydrogenated fat (Crisco)				10	10	10	10	10
Carotene in corn oil (3:1000)				0.3	0.3	0.3	0.3	0.3
$CaCO_3$	3.0	1.5	0.5	3.0	1.5	0.5	3.0	1.0
$NaH_2PO_4 \cdot H_2O$		2.0	3.5		1.8	3.3		1.8
Phytin							1.5	1.5

The edestin was of "technical" grade.[2] The percentage composition of salts no. 4 was as follows: $NaHCO_3$, 37.21; $MgSO_4$, 10.14; KCl, 48.53; $FeCl_3 \cdot 6\ H_2O$, 3.21; $CuSO_4 \cdot 5\ H_2O$, 0.44; $MnSO_4 \cdot 2\ H_2O$, 0.14; $ZnSO_4 \cdot 7\ H_2O$, 0.30 and KI, 0.02. Phytin, containing 14.2% phosphorus, was prepared from wheat bran, according to Boutwell's ('17) procedure. It was free from inorganic phosphorus.

Eight basal diets were used (table 1). In all experimental diets citrates were added by replacement of equal weights of sucrose. Diets 1a-3a (table 2) contained 2% citrate mixture (citric acid 19.2 gm., sodium citrate 28.6 gm.). Diets 4a-8a (table 2) contained 4% of the same mixture. The citrate mixtures, fed at these levels, caused no appreciable effect on the food consumption.

RESULTS

Table 2 summarizes the data. In order to conserve space only mean values are presented, with calculations of the statistical significance of the mean differences expressed as t (Snedecor, '38, p. 57).

It is shown that a citric acid-sodium citrate mixture at the moderately low level of 2% permits improved ossification when fed with the high calcium-low phosphorus Steenbock-Black ricketogenic diet (diets 1 and 1a). The content of bone ash in the citrate fed rats possibly was raised significantly ($t = 1.62$). However, t would have to be 2.30, or higher, to be unequivocally significant. With a moderate amount of calcium and phosphorus (diets 2 and 2a) the percent bone ash was even lower in the citrate-fed rats than in the controls, but the difference may not be significant ($t = 1.95$). The presence of citrate in the basal diet made rich in phosphorus and poor in calcium (diets 3 and 3a) was without effect on the total ash and percent ash. It may be significant, however, that all of the rats fed citrate lost weight while one-half (four) of the controls made small gains in weight, although the average for all the controls showed a slight loss of weight.

[2] Obtained from the Arthur H. Thomas Company.

TABLE 2

Calcifying action of a citric acid-sodium citrate mixture in diets containing (1) corn, (2) purified components and (3) purified components plus phytin

DIET			CITRATE MIXTURE	NO. OF RATS	WEIGHT INCREASE	BONE ASH					
						Mg.			per cent		
No.	Ca	P				Mean	Deviation from mean	t	Mean	Deviation from mean	t
Experiment 1 (corn)											
	%	%	%		gm.						
1	1.25	0.25	—	7	18	54.3	8.2		37.7	1.8	
1a	1.25	0.25	2	7	19	64.8	8.7	1.62	42.3	1.7	3.61
2	0.65	0.75	—	8	7	84.3	8.4		53.2	1.2	
2a	0.65	0.75	2	8	3	82.3	11.4	0.29	51.7	1.1	1.95
3	0.25	1.05	—	8	—1	65.9	6.6		46.7	1.5	
3a	0.25	1.05	2	8	—6	68.1	6.6	0.54	47.1	1.0	0.49
Experiment 2 (purified components)											
4	1.25	0.20	—	7	32	77.9	9.3		43.5	1.3	
4a	1.25	0.20	4	7	28	81.3	13.8	0.44	44.8	1.7	1.50
5	0.65	0.65	—	7	24	103.7	9.7		54.9	0.8	
5a	0.65	0.65	4	7	20	95.3	8.6	1.51	53.4	1.1	2.31
6	0.25	0.95	—	8	4	66.6	3.6		50.1	0.6	
6a	0.25	0.95	4	8	6	67.2	4.7	0.23	50.3	1.7	0.25
Experiment 3 (purified components plus phytin)											
7	1.25	0.40	—	8	35	90.8	8.9		43.8	1.7	
7a	1.25	0.40	4	8	36	110.3	11.8	2.87	48.1	0.9	5.00
8	0.45	0.80	—	8	20	103.1	14.3		50.6	1.0	
8a	0.45	0.80	4	8	20	102.7	12.0	0.04	51.9	1.2	1.92

The favorable effect of citrates observed with diet 1a did not occur when a high calcium-low phosphorus purified diet was fed (diets 4 and 4a), even though it contained twice as much citrate. Also, there was no effect with moderate calcium-moderate phosphorus (5 and 5a) or low calcium-high phosphorus diets (6 and 6a). It should be noted that the total bone ash of rats fed the high calcium-low phosphorus corn diets (1 and 1a) is definitely lower than that of rats fed the high calcium-low phosphorus purified diets (4 and 4a). Likewise, the former rats gained less weight than the latter. By all the criteria employed, rats receiving the corn diets developed less favorably than animals fed the corresponding purified diets containing approximately the same content of calcium but less phosphorus. In planning these rations no attempt was made to render the purified diets equivalent to the corn diets with respect to total phosphorus content owing to the variable biological availability of phosphorus in the latter.

On the basis of results from these experiments it appeared evident that citrate in purified diets was without significant effect on ossification whatever the levels of calcium and phosphorus, and in rations composed principally of corn the beneficial effects on bones occurred only when there was an excess of calcium carbonate. Hence it was of interest to observe the effect of adding the principal phosphorus compound of corn, phytin, to purified diets containing citrate. There is no doubt that citrate was beneficial in the presence of an excess of calcium (diets 7 and 7a). Both the total bone ash and the per cent ash were significantly increased. But with moderate levels of calcium and phosphorus (diets 8 and 8a) neither the total ash nor the per cent of ash were affected by the presence of citrate.

DISCUSSION

The data reported here are in general conformity with the findings of previous investigators as respects the beneficial effects of citrates on ossification in rats fed a high calcium-low phosphorus cereal ration.

However, the citrate mixtures were fed at a much lower level than in the experiments of Shohl ('37), Hamilton and Dewar ('37) and Hathaway and Meyer ('39). Their diets contained as much as 20% or more citrate. But apparently they experienced little difficulty in getting the animals to eat the diets since Hathaway and Meyer ('39), who reported food consumption data, noted no appreciable depression of weight increase or food consumption. Possibly higher levels of citrate in our diets would have produced more exaggerated changes in the bones affected but it does not seem probable, in view of the indicated effect on phytin, that the non-significant values would have become significant.

With due regard for differences in experimental technic, our findings appear to be at variance with those of Shohl ('37), with respect to the effect of citrates on rats fed a low calcium-high phosphorus cereal diet. Shohl depended principally upon histological evidence while in the studies reported here the criteria used were the total bone ash and the per cent ash (from fat-free bones).

It is possible that Shohl's animals did not respond to citrate by an increase in ossification since the effect on total bone ash is not given. Indeed, as pointed out by Hathaway and Meyer ('39), "His (Shohl) table 2 shows that with diets S and K, slightly basic basal diets, the addition of the citric acid plus sodium citrate cured the rickets (by histological and x-ray diagnosis) but materially lowered the bone ash values, in diet S by 4%, in diet K by 9%." It is possible that our citrate fed rats (table 2, diet 3a) did not develop rickets, on the basis of histological criteria, although the failure of ossification was as great as in the control animals not fed citrate. Therefore, it is possible that there is no actual variance between Shohl's findings and those reported here since he was dealing with rickets only, and our data are confined to evidence on the mineralization of bone. Hathaway and Meyer ('39) have commented adequately on the relative merits of these technics, as respects the appraisal of calcifying agents, pointing out that, "improvement in bone ash indicates improved calcification

throughout the bone and 'cure of rickets' with continuance of osteoporosis does not materially improve the condition of the animals.'' Chick, Korenchevsky and Roscoe ('26) have shown that chemical analyses of bones furnish a better criterion of defective calcification than histological examination alone.

Hamilton and Dewar ('37) suggested that, ''The beneficial effect of citrate and tartrate is probably exerted largely in the intestinal tract and is possibly due to a decrease in available calcium.'' The inapplicability of this suggested explanation is shown by the data reported here since citrate was without effect in high calcium-low phosphorus diets that did not contain phytin, either as such or in corn. If the effect was simply a matter of preventing phosphate precipitation in the intestinal tract, through binding of the excess calcium, calcification should have been improved in our high calcium-low phosphorus purified diet.

It seems probable that the explanation must be sought in the effect of citrates, directly or indirectly, on the biological availability of phosphorus complexes in the basal diets, as indicated previously. The findings of Lowe and Steenbock ('36a) would seem to be extremely pertinent in this respect since they clearly demonstrated that phytin phosphorus is rendered less available in the intestinal tracts of rats by the presence of excess calcium carbonate. It is a plausible assumption, therefore, that the beneficial effect of citrates is mediated through the formation of a calcium complex which interferes less, if at all, with the utilization of phosphorus in phytin, and perhaps other complexes of relatively low biological availability. That the effect is not simply one of producing a more favorable reaction (pH) for phytase activity is not very probable since, as observed by Shohl ('37), some other organic acids such as acetic, lactic, malic, malonic and succinic, and their sodium salts, do not improve the condition of the bones.

In view of the evidence reported here it would seem that there is need for caution in applying to problems of human

rickets the findings based on rickets prevention in animals fed experimental high calcium-low phosphorus cereal rations containing citrate. If the principal effect of citrates on calcification is as postulated here, there seems to be scarcely any basis in fact for the opinion that citrate administration might constitute an effective means of preventing human rickets, or of exerting any significant antiricketic effect. This is a plausible conclusion since the diets of infants and children, although generally containing considerable quantities of phytin, are not characterized by excesses of calcium. The fact cannot be ignored, however, that Shohl and Butler ('39) have concluded, on the basis of preliminary results from two cases, that a citric acid-sodium citrate mixture "not only offers an adjunct to accepted measures for the treatment of infantile rickets, but also may have application to other types of disorders of calcium and phosphorus metabolism." Obviously this is an insufficient number of cases to warrant reliance on the findings, particularly since complicating factors intervened as Shohl and Butler have recognized. It is necessary to mention that Albright et al. ('40), in a report on one case of "nephrocalcinosis with rickets and dwarfism", conclude that administration of a citric acid-sodium citrate mixture was of benefit in treatment of the disease. However, in this instance the primary metabolic disturbance appeared to be an acidosis which resulted in loss of base, including calcium. This eventuated in 'low phosphorus' rickets. Hence, as Albright et al. have suggested, the benefit probably derived from the sodium and not the citrate.

Chaney and Blunt ('25) observed a definite increase in calcium and phosphorus retention of girls fed large amounts of orange juice. This has been cited as possible evidence of the favorable effect of citrates on calcification (Hamilton and Dewar, '37; Shohl and Butler, '39). It should be recognized, however, that the control diet was deficient in ascorbic acid and possibly other essential nutrients. Since marked nitrogen retention occurred also, when orange juice was fed, it is at least equally plausible that the favorable mineral retention is

attributable to the ascorbic acid ingestion. However, the finding of Lanford ('39), that rats on a whole wheat-milk powder diet retain more calcium when fed orange juice, is not so readily explained on the basis of ascorbic acid supplementation, because the rat, presumably, is not benefited by dietary ascorbic acid. Since the Ca:P ratio of Lanford's diet was not excessive, her results do not appear explicable on the same basis as the data reported here. Whether citrate alone would yield the same effect with Lanford's diet must be determined before it can be concluded that her findings and those reported here are contradictory.

The data presented in this paper, and the discussion of other findings, emphasize again the difficulties that may arise in the exclusive use of cereal rations in the study of rickets and other problems concerned with calcium and phosphorus utilization. Further investigation is needed to reveal the mechanism whereby citrate promotes better ossification when the diet contains phytin and an excess of calcium.

SUMMARY

Studies have been made of the effect on bone ash of young rats fed (a) cereal rations and (b) "purified" rations containing a mixture of citric acid and sodium citrate and different amounts of calcium and phosphorus.

The finding of other investigators has been confirmed that citrates promote calcification in the bones when added to cereal rations high in calcium and low in phosphorus.

With a purified ration citrate does not promote calcification when the calcium level is high and phosphorus is low.

Citrates have no appreciable effect on calcification when the diet contains moderate levels of calcium and phosphorus, or is low in calcium and high in phosphorus, irrespective of whether a cereal ration or a purified one is used.

Citrates added to a purified ration containing phytin cause a definite increase in calcification if the Ca:P ratio is high, but there is no effect with a moderate Ca:P ratio.

It is indicated that a beneficial effect of citrates in ricketogenic diets does not occur unless there is a high Ca:P ratio and appreciable quantities of phytin, or perhaps other phosphorus-containing complexes of low biological availability.

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A BIOLOGICAL ASSAY OF RIBOFLAVIN IN THE LIVER OF THE COW, CALF, SHEEP, LAMB, AND HOG [1]

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TWO FIGURES

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Riboflavin is found rather abundantly in a variety of foodstuffs including milk, lean meat, liver, and the green growing parts of plants. Since information is lacking upon the riboflavin content of other kinds of liver than beef and pork (Daniel and Munsell, '37; Hoagland and Snider, '30), this study was undertaken to obtain additional information regarding the riboflavin values of livers from various animals.

PROCEDURE

Livers for these experiments were purchased in August, September, October, and again in December, 1936, at local retail markets which had obtained their meats from packing plants in Kansas City. In the fall one sample each was purchased of beef, calf, pork, and lamb livers. Each sample represented two to six animals. In December, a second set of samples was purchased and sheep liver was included.

The thick blood vessel walls in the liver and any inedible portions that would ordinarily be discarded if the liver were to be eaten were removed and the raw livers run through a food chopper having a fine blade; after mixing they were put through the chopper a second time. They were then placed in glass containers and stored at a temperature below 0°C.

[1] Contribution no. 88, Department of Home Economics.

Small amounts were removed as needed for feeding experiments.

The Bourquin-Sherman ('31) method was used and the tests run in conjunction with riboflavin [2] as a standard. Booher, Blodgett and Page ('34) and also Bisbey and Sherman ('35) and Bessey ('38) have shown this method of assay to be suitable for quantitative determinations of riboflavin in foods.

The casein was prepared according to the method suggested by Ellis ('32). The Osborne and Mendel salt mixture was made by the time-saving method suggested by Wesson ('32), and the butterfat was melted and filtered at 45° to 55°C.

One hundred and thirty-six albino rats of The Wistar Institute strain, raised on the diet described by Sherman and Crocker ('22), were used in this experiment. They were placed on the riboflavin-free diet at 3 weeks of age if they reached an approximate weight of 38 to 40 gm. In our laboratory rats 3 weeks old have been found to be more satisfactory than 28-day old animals. This is in accord with the work of Lassen ('36). Toward the end of the depletion period the rats were weighed daily, and when the weights were judged to be stationary, the animals were put in individual cages and supplements of test material allotted. The average depletion period was 14 days and the average weight at the time of depletion was 48 gm. with a range of 40 to 56 gm.

The group of animals on each sample of liver generally included ten or more individuals, and was made up approximately of the same number of males and females. Nine groups received the test livers, two received riboflavin, and one received only the basal diet and water.

The animals receiving liver were given 200 mg. twice weekly. They liked the liver and consumed it within a few seconds so that there was no difficulty in getting quantitative consumption of the test food. A rat from each litter used in the experiment was given 2.5 or 5.0 mcg. of riboflavin and one was given no supplement. The riboflavin solution was made up to 1 mg. per 100 ml. of distilled water and was fed to the rats at two levels,

[2] "Lactoflavin, PX grade" from the Borden Company was used.

namely 2.5 and 5.0 mcg., daily. The solution was dropped directly into the mouths of the animals from a 1-ml. syringe graduated in hundredths.

During the test period, the animals were weighed each week and records were made of the amount of food consumed.

TABLE 1

Riboflavin content of liver from various animal sources collected during the fall and winter seasons, as revealed by rat feeding experiments

SUPPLEMENT FED	DAILY PORTION FED	NUMBER RATS	AVERAGE DEPLETION PERIOD	AVERAGE INITIAL WEIGHT	AVERAGE GAIN IN WEIGHT	ESTIMATED RIBOFLAVIN PER 100 GM.
	mg.		*days*	*gm.*	*gm.*	*micrograms*
Lamb liver						
fall	66.7	15	14	45	22	4950
winter	66.7	10	13	47	24	5400
Mutton liver						
winter	66.7	10	16	49	20	4350
Calf liver						
fall	66.7	10	14	46	15	3450
winter	66.7	12	16	50	20	4350
Beef liver						
fall	66.7	12	14	46	13	2850
winter	66.7	8	14	47	16	3450
Pork liver						
fall	66.7	15	14	47	12	2700
winter	66.7	11	15	48	12	2700
None	0	16	15	50	—1	
	micrograms					
Riboflavin	2.5	10	13	46	17	
Riboflavin	5.0	9	14	49	33	

Observations were made upon the activity and appearance of an animal at the end of the experimental period and any unusual behavior during the test period was recorded. The record of any animal suspected of coprophagy was not used in the final summary. This accounts for the smaller number of animals in some groups.

FINDINGS

A summary of the data from the feeding experiments is given in table 1. At the end of the 8-week period, the test ani-

mals showed a gain of 12 to 24 gm. of weight. The negative control animals lost 1 gm. of weight. The control group given 2.5 mcg. riboflavin per day showed an average gain of 17 gm., and the group fed 5.0 mcg., an average gain of 33 gm. The gains of these groups compared favorably with those of Ansbacher's (Ansbacher, Supplee and Bender, '36) experimental animals.

The positive control groups and the test animals fed livers containing highest riboflavin content had bright eyes, glossy

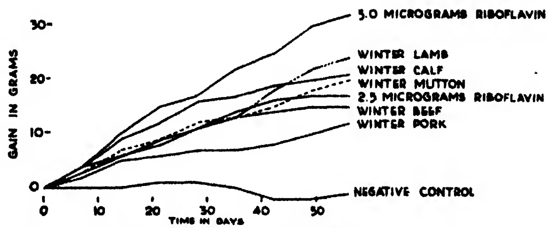

Fig. 1 Growth curves of rats on riboflavin-free diet plus animal livers collected during the winter season compared with 0.0, 2.5, and 5.0 mcg. of riboflavin fed daily to the control groups.

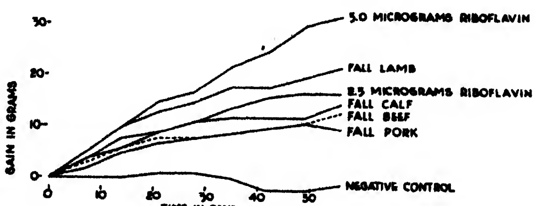

Fig. 2 Growth curves of rats on riboflavin-free diet plus animal livers collected during the fall season compared with 0.0, 2.5, and 5.0 mcg. of riboflavin fed daily to the control groups.

fur, good muscular tone, and a normal appetite. They were vigorous in their activity and seemed to be satisfied. The test animals fed pork liver as a source of riboflavin were vicious, restless, and lighter in weight, with shaggy fur and dull eyes. The negative control group lacked appetite and good muscular tone, and were light in weight; their general appearance was extremely poor, as the fur was rough, the eyes listless, the posture hump-backed, and movements sluggish.

The lamb liver showed the greatest potentiality for improved growth in rats while mutton was second; calf, third; beef, fourth; and pork, lowest as shown in table 1 and figures 1 and 2. The riboflavin values for the different samples were estimated by interpolating between the gains in weights by the groups of rats on 2.5 and 5.0 mcg. of riboflavin. Where the groups of animals on liver showed weight gains less than either one of the groups of riboflavin, values were estimated by extrapolation on the basis of an assumed curve of response.

SUMMARY AND CONCLUSIONS

1. Samples of beef, calf, lamb, and pork livers were purchased in the early fall, then again in early winter with the addition of mutton livers. Each of the five liver samples represented two to six animals.

2. The assay for riboflavin was made according to the procedure of Bourquin and Sherman. Riboflavin fed at 0.0, 2.5, and 5.0 mcg. levels daily was used as a standard. The riboflavin content of the livers was estimated by comparing the gain in weights of the rats given the liver supplements with those given the riboflavin supplement.

3. Lamb liver, which showed the greatest potentiality for gain in weight in rats, was estimated to contain 4950–5400 mcg. per 100 gm.; mutton liver, 4350 mcg.; calf liver, 3450–4350 mcg., and beef liver, 2850–3450 mcg. per 100 gm. Pork liver, estimated to contain 2700 mcg. per 100 gm., ranked lowest in riboflavin value.

4. Liver samples purchased in the winter season were slightly higher in riboflavin value than samples purchased in the fall.

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THE RIBOFLAVIN CONTENT OF CERTAIN HAYS AND GRASSES[1]

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Since riboflavin is part of the vitamin B complex and is necessary for the growth and normal functioning of animals, a further interest has developed in studying its natural sources and the conditions under which it is preserved or destroyed in natural products.

Hays and grasses are used, more or less, in the feeding of every type of farm animal. Poultry especially require a liberal quantity of green, leafy hay or meal in order to maintain a high level of production and hatchability. Because of the extensive use of alfalfa leaf meal in poultry rations to supply the riboflavin, it was thought advisable to make a study of the influence of time of cutting and method of curing on the riboflavin content of some common hay plants and some grasses not commonly used commercially.

EXPERIMENTAL PROCEDURE

The plants

The description of the plants, time of cutting, and method of curing are given in table 2. Those plants cured in the sun were exposed on a western slope between the hours of 9:00 A.M. and 4:00 P.M. for a total of 48 hours. This is much longer than hay is ordinarily cured, but it was thought desirable to give the samples extreme exposure in order to note the effect of sunlight on the riboflavin content. Neither dew nor rain was

[1] Published with the permission of the Director of the Ohio Agricultural Experiment Station, Wooster.

allowed to fall on the samples. Practically all of the green color was destroyed during this exposure. After drying, the hays were ground to a very fine powder in a ball mill and stored at refrigeration temperature until time for assay.

Biological assay

The rat growth method was used for the assay of the samples for riboflavin. The rats were confined in screen-bottom wire cages when weighing 35 to 45 gm. and fed the basal ration. The basal ration, consisting of vitamin-free casein 20 parts, sucrose 71, salt mixture 4, Crisco 3, and cod

TABLE 1

Growth rate of rats. Weight increase on varying amounts of riboflavin; average of four experiments

SUPPLEMENT	NUMBER OF ANIMALS		AMOUNT FED DAILY	AVERAGE INCREASE IN WEIGHT 6 WEEKS	AVERAGE INCREASE IN WEIGHT OVER NEGATIVE CONTROLS
	♂	♀	*micrograms*	*gm.*	*gm.*
Positive control, synthetic riboflavin (Merck)	16	16	2.5	32	25
	16	16	5.0	55	48
	16	16	10.0	82	75
	16	16	20.0	106	99
	16	16	40.0	121	114
Negative controls	12	12	0.0	7	—

liver oil 2 parts respectively, was supplemented with daily addenda of 10 mcg. of thiamin [2] and a water-soluble fraction of yeast from which the greater part of the riboflavin had been removed by treatment with fuller's earth (see negative controls tables 1 and 2). The amount of this yeast extract fed daily was equivalent to 1.5 gm. dried yeast. This same amount of yeast extract when supplemented with adequate amounts of riboflavin and thiamin produced excellent growth, i.e., 18 to 20 gm. per week, and the same yeast extract, in amount of 0.25 cc. (0.25 gm. yeast), also cured acrodermatitis of rats.

[2] From the Merck Company, Rahway, New Jersey.

When the rats had ceased to gain in weight they were transferred to individual cages and the finely ground samples of the test hay or grass were fed, in separate dishes, as the source of riboflavin. The supplemental feeding was continued for 6 weeks.

The curve obtained by plotting the weight increment due to the feeding of different levels of pure riboflavin (minus the

TABLE 2

Riboflavin content of hays and grasses

FEED SUPPLEMENTS	TIME OF CUTTING	METHOD	NUMBER OF ANIMALS	AMOUNT FED DAILY	AVERAGE INCREASE IN WEIGHT 6 WEEKS	AVERAGE INCREASE IN WEIGHT OVER NEGATIVE CONTROLS	ESTIMATED RIBOFLAVIN CONTENT PER GRAM
				mg.		*gm*	
Oat plant	June 4: 8 to 10 in. high; before jointing	Subdued light	4 4	75	32	20	26
Wheat plant	May 21: 10 to 12 in. high; jointing	Subdued light	4 4	75	27	15	17
Bluegrass	May 21: beginning to head	Subdued light	4 4	200	32	20	10
Alfalfa	May 21: before bloom	Subdued light	4 4	200	34	32	16
Alfalfa	May 21: before bloom	48 hours strong sunshine	4 4	200	37	25	12
Alfalfa	June 7: beginning to bloom	Subdued light	4 4	300	48	36	12
Alfalfa	June 7: beginning to bloom	48 hours strong sunshine	4 4	300	40	28	9
Timothy	June 1: before heading	Subdued light	4 4	300	61	49	17
Timothy	June 10: in head before blooming	Subdued light	4 4	300	52	40	12
Negative control			4 4		12		

negative controls) was used to calculate the riboflavin content of the products under study. This method is somewhat similar to that of Supplee and associates ('39).

RESULTS AND DISCUSSION

The results of the assays of the different samples are given in table 2. The differences in the riboflavin content of the oat, wheat, and bluegrass plants, as noted in table 2, may be accounted for by a difference in the maturity of the plants, as shown under the heading, "Time of cutting," and more completely in a previous publication (Hunt, Record and Bethke, '36). There is no evidence in this study and in the previous one that any one plant of this type is superior to another as a source of riboflavin. The data do show that plants of this type are a very good source of riboflavin and offer possibilities as a natural source of riboflavin for practical purposes.

The assays of the alfalfa plant show that the riboflavin content per unit of weight decreased as the plant matured. There was a loss of about 25% due to a difference of about 2 to 3 weeks in maturity. The results from the alfalfa cut May 21 and June 7 are in close agreement with the data presented by Heiman and associates ('38) in which they compared the vitamin G (riboflavin) content of alfalfa hay cut when 10 and 24 inches high. The riboflavin content of the alfalfa cut May 21 is also in agreement with that found by Norris and associates ('36) who obtained a value of 16 chick units of vitamin G per gram of dehydrated alfalfa leaf meal (a chick unit is equivalent to 1 mcg. of riboflavin). Jukes ('37) obtained a value of 8 rat units of vitamin G per gram for dehydrated alfalfa meal. Hunt, Record and Bethke ('35, '36) obtained values of 6 to 12 units of vitamin G per gram of alfalfa hay. Data from this laboratory (unpublished) show that 1 rat unit (Bourquin-Sherman) is equivalent to 2 mcg. of riboflavin. Applying these data would give a value of 16 mcg. for Juke's sample and 12 to 24 mcg. for those obtained from this laboratory, whereas Supplee and associates ('39) obtained a value of 28 mcg. for

alfalfa meal. The apparent difference in the results obtained in these experiments and those obtained in a previous one is probably due to a difference in the maturity of the plants used or the percentage of leaf content in the sample.

The results obtained from alfalfa hays exposed to bright sunshine for 48 hours show that there is a loss of about 25% of their riboflavin content. That there was no greater loss may mean that part of the riboflavin may exist in these plants in the combined form. The data show, however, that hays used as a source of riboflavin should be exposed to sunshine for as short a time as is consistent with good practice.

The riboflavin content per unit of weight of the timothy plant also decreased as the plant matured. This loss is about 33% for a difference of about 10 days in maturity. The assays show that young timothy plants, as well as oats, wheat, and bluegrass, may contain as high a riboflavin content as early cut alfalfa.

The data shown in table 2 suggest that hays intended to supply the riboflavin requirements of animals should be cut before or not later than time of blooming and that the product should not be exposed to strong sunshine any longer than is necessary for proper curing. Curing in windrows or cocks, as is now done in good practice, will accomplish this.

SUMMARY AND CONCLUSION

The riboflavin content, per unit of weight, of the alfalfa and timothy plants used in this study decreased as the plants matured. Oat, wheat, and bluegrass plants were studied only at one stage of maturity.

Alfalfa plants exposed to strong sunshine for 48 hours after cutting suffered a loss of about 25% of their riboflavin content.

Plants such as oats, wheat, bluegrass, and timothy offer a very good source of riboflavin in the early stages of development, and a more extended use of such products as a practical source of riboflavin is suggested.

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HISTOLOGICAL STUDIES OF THE TISSUES OF RATS FED A DIET EXTREMELY LOW IN PHOSPHORUS [1]

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ONE PLATE (SIX FIGURES)

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Although an extensive literature has grown up dealing with the histological alterations in the skeletal system of rats in which high calcium-low phosphorus rickets had been produced, the other tissues of such animals for the most part have been neglected. Furthermore, no appreciable attention has been given to the microscopic study of tissues from animals on diets low in phosphorus but containing a normal amount of calcium, vitamin D and other nutrients. Therefore, it is of interest to examine the tissues of animals on a diet extremely deficient in phosphorus in order to determine whether or not extra-skeletal histo-pathological changes can be detected.

EXPERIMENTAL

Material and methods

The basal experimental diet used in these studies was reported by Day and McCollum ('39). Its percentage composition was as follows: edestin 16.0, gelatin 4.0, sucrose 61.2, salts (P-free) 4.0, choline hydrochloride in sucrose (1:9) 1.0, cystine in sucrose (1:19) 1.0, hydrogenated fat [2] 10.0, carotene in corn oil (3:1000) 0.3, viosterol 15 drops (3800 I. U.) per

[1] This study was aided by a grant from the Rockefeller Foundation.

[2] Crisco was used.

kilogram, and vitamin B complex 2.5%. This diet contained 0.017% phosphorus and 0.4% calcium and was designated diet 16 by Day and McCollum ('39); when supplemented with phosphorus (0.27%), it became diet 17.

A total of seventy-three rats were used. They were placed on the diets when they averaged about 50 gm. in weight. Two consecutive series were studied. Series I consisted of fifty-seven animals about equally distributed as to sex. They were fed ad libitum. Twenty-eight animals received diet 16, while twenty-nine received diet 17. Six animals (three experimental and three controls) were killed at weekly intervals for 7 weeks. The remaining rats came to autopsy at various times during the eighth and ninth weeks. Series II consisted of sixteen animals, six of which were placed on diet 16 while ten were given diet 17 but the total intake of these control animals was restricted so that it was essentially equal to that of the phosphorus deficient group. With such paired feeding it was believed that the effect of partial inanition and its influence on the tissues could be satisfactorily judged. These animals came to autopsy from the forty-eighth to the sixty-seventh day.

The animals were killed with ether and autopsies performed immediately. Tissues were placed in Zenker-formol and in formalin alone. After dehydration the tissues were imbedded in paraffin, cut and stained with haematoxylin and eosin. Other special stains were used when indicated. To study the changes in the bones, the distal end of the femur was decalcified in 2% nitric acid and imbedded in paraffin while after decalcification the proximal end of the tibia was imbedded in celloidin. X-rays of the skeleton were taken at varying intervals during the experiments.

Since it seemed inadvisable to examine all the tissues in every animal, an initial study was made of all the organs of animals in series I from the seventh week on. Then those tissues showing changes were studied in the earlier periods and the development of such changes was traced. The same tissues of the rats in series II were likewise examined in

order to determine whether phosphorus deficiency alone or merely restriction of the diet was the factor producing the alterations noted.

Gross appearance of the animals

In the animals of series I it was quite apparent after the second week which ones were on diet 16 (low P). These animals were much smaller than the control rats (Day and McCollum, '39). The coats of the deficient animals were rough and unkempt. The skeletal system became greatly rarefied. The bones of the phosphorus low rats were soft and cut with ease. Toward the end of survival, brown encrustations were noted about the nares. There was a great decrease in adipose tissue in the phosphorus deficient animals. The subcutaneous fat and that usually seen in the abdomen and neck was either sparse or entirely lacking. This was noticeable from the second week on. Especially striking was the decrease in perirenal fat and that about the uterine horns. The size of the various organs was decidedly reduced in the deficient animals and seemed to parallel the size of the whole animal. Organ weights were not determined. Jackson and Carleton ('23) have reported such a study in experimental rickets in the rat.

More impressive still were the changes noted in the form of the thorax. The bones became progressively more pliable and because of the respiratory movements the ribs became bowed at the costo-chondral junctions. Thus the capacity of the thorax became less and less due to this sinking in of the lateral walls. This could be quantitatively determined by measuring the amount of water such a thoracic cavity would hold. At 42 days the thorax from a deficient animal had a capacity of 4 cc. while that of a control was 10 cc. Owing to the softening of the ribs there was a marked collapse of the lungs with apparent respiratory difficulty. These findings in the thorax are identical with those observed by Park and Howland ('21) in children with severe rickets.

In contrast to the differences in size between the rats on diets 16 and 17 of series I were the findings in the animals of series II. These latter animals showed little variation as to size. Externally there were few distinguishing features. The bones of the phosphorus low group were more easily bent and cut. The thorax, too, was deformed as was seen in the previous group. However, due to inadequate food intake the thoraces of the control group were small though their capacity was not so reduced as in the deficient animals. There was at autopsy perhaps a little more adipose tissue in the controls but even here the change was not very striking.

MICROSCOPIC FINDINGS

Skeletal system. In the animals of series I changes indicative of rickets were present in the bones after they had been on diet 16 for only 7 days. At this time, there was a broad plate of epiphyseal cartilage due to an increase in number of fully grown cartilage cells. The trabeculae at the cartilage-shaft junction showed osteoid borders and there was a decrease in calcified matrix substance between the rows of cartilage cells. From this time on the ricketic change became progressively marked until the forty-ninth day. At this time due to the slowing of growth the changes were much less conspicuous. X-rays taken at 14 days and at various intervals thereafter seemed to verify the changes that were taking place in the bones. It seems unnecessary to detail the bone changes since they proved to be qualitatively identical with those observed in the classical studies of experimental high calcium-low phosphorus rickets made by Shipley, Park, McCollum and Simmonds ('21) and Sherman and Pappenheimer ('21) in the rat. They also confirm the more recent detailed studies on rickets in the rat by Dodds and Cameron ('34).

In the animals of series II, ricketic changes were also produced in the deficient rats and no such changes were found in the controls on the restricted intake. However, the effect of partial inanition by evidence of a slowing of bone growth was noted.

Even in the presence of moderate vitamin D ricketic changes were produced within 7 days. This is not an unexpected finding, since Querido ('35) has shown that rats on a cereal diet containing 0.35% calcium and 0.12% phosphorus devel-

oped rickets which could not be completely prevented by moderate amounts of vitamin D (in the range employed in our studies). However, up to the present experiments, purified diets extremely low in phosphorus content have not been studied and it has been necessary to greatly restrict the intake of vitamin D in order to produce rickets by the usual methods.

Teeth. A few sections of teeth were studied but due to technical difficulties not enough were examined to warrant any conclusions.

Heart and blood vessels. No abnormalities were noted in the deficient animals.

Lungs. In the first week the lungs of the animals of series I were unaffected. However, from 14 days on, as the difference in size of the control and deficient rats increased, differences in the lungs were also noted. By 21 days, this difference was obvious. The pulmonary alveoli of the control animals were large and fully expanded, while the lungs of the deficient rats had small, incompletely expanded alveoli, some being completely collapsed. This atelectasis was, of course, due to the continuous decrease in the capacity of the thoracic cavity which, as the deficiency advanced, made it progressively harder for the lungs to expand. This interference with respiration and aeration of the blood possibly contributed to the death of the animals. How much this respiratory difficulty contributed to a lessening of activity with resulting decrease in food intake and weight loss is of course mere speculation.

In series II the thoracic differences were present but less striking since the thoracic capacity of the diet restricted controls was much smaller than in the ad libitum controls.

Thymus. In animals of series I autopsied in the first and second weeks, no differences could be detected between the control and deficient rats. By the third week, however, the difference was striking enough. The cortical portion of the thymus had decreased in thickness and this was evidently due to a loss of lymphocytes since these cells were fewer and more loosely packed than in the controls. The medulla, too, was a little smaller. From then on there was a progressive shrinkage of the thymus in the deficient animals, both medullary and cortical regions taking part in the change, so that in the eighth and ninth weeks only vestiges of thymic tissue remained. By this time, too, the thymus of animals on the ad libitum control diet had begun to become smaller due probably to age.

In the paired feeding series no appreciable difference could be found between the two groups. It appears, therefore, that the changes which the organs exhibited were due to inanition alone. This has been noted by several observers (Jackson, '25).

Spleen. After several weeks on the diet, the spleens of the control animals in series I tended to be larger than those of the phosphorus deficient rats. Microscopic study of the spleens of the latter animals revealed smaller Malpighian bodies and what was taken to be an increase in hemosiderin. However, the Prussian blue reaction revealed that each contained about the same amount of pigment. In our experience the rat's spleen normally contains a good deal of pigment, more than other laboratory animals such as the rabbit and guinea pig. It does not appear therefore, that there was any severe blood destruction in the deficient animals and, as is pointed out below, the kidneys contained no pigment either.

Liver, gastro-intestinal tract and pancreas. No differences were noted between the deficient animals and their controls.

Adrenals. In series I the adrenal tissue of the deficient animals showed a decided decrease in the thickness of the cortex. All three cortical layers were affected. Frozen sections stained for fat with Scharlach R, showed decidedly less lipid material in the phosphorus low animals. The decrease was mainly in the fascicular and reticular layers. The changes observed seemed identical with those described by Jackson ('19) in chronic inanition in rats. That the changes observed by us also were due to inanition is suggested by the fact that the adrenals of the paired feeding group showed no differences histologically.

Kidneys. Except for the differences in the size of the various elements, the kidneys of series I showed no appreciable changes. It is significant that no renal calculi, such as have been recently described by Schneider and Steenbock ('39, '40), were observed in any of our phosphorus deficient rats. The above authors' diet contained 0.04% phosphorus and 0.57% calcium and they state that single calculi were observed as early as the seventh week but large numbers of uroliths were not noted until the twentieth week. Our experiments differ from theirs in that our diet contained less calcium and phosphorus than the one they employed and, moreover, our animals lived at the longest 68 days. Bluish-staining "calcified" material was found in the renal tubules of one of our control

animals, and in our experience, such deposits are not uncommon in the rat, especially in older animals. No iron pigment was found in the tubular epithelium.

Urinary bladder and ureter. The mucosa of the urinary passages showed no differences in the deficient and control animals.

Testes. In series I the testes of the deficient and control animals autopsied after 7 days were essentially the same. Spermatogonia and spermatocytes with many mitotic figures were seen. There were a few spermatids but no mature spermatozoa. At the end of 14 days the picture in the deficient animals was unchanged while the tubules of the controls were larger and there were large numbers of spermatids. After 21 days, active spermatogenesis was taking place in the controls with numerous spermatozoa, while the deficient rats remained essentially unchanged. After 35 days, however, a few spermatozoa could be found in the tubules of the deficient rats. The difference in tubular size of the two groups was striking from this time on. Spermatogenesis continued in the phosphorus low animals until the forty-ninth day when it practically ceased. There was almost a complete lack of spermatids and only a few spermatozoa. Giant cells were also seen in the lumina of the tubules. In the paired feeding series there was little if any difference between the two groups. Spermatogenesis was taking place in a few tubules but it was not very active. The tubules were small, thus it seemed evident that the differences noted in series I were due to inanition and they resemble the changes which have been so well described by Siperstein ('21).

Accessory sex organs. No changes in the prostates, seminal vesicles and coagulating glands were observed in series I during the first 3 weeks. After 28 days, however, there was a definite increase in the size of these organs in the control group. There was evidence of an increased secretion and the epithelial cells became more columnar. In the ensuing weeks there was the same difference in size of these structures. The epithelium of the deficient animals became more columnar yet never appeared as active as that in the controls. By the fifty-sixth day, the epithelium had become less columnar in the deficient animals and there were fewer vacuoles in the individual cells. In the animals of series II differences between the deficient rats and controls were not apparent. The glands and seminal vesicles were small and the epithelium was

low columnar in appearance with few vacuoles. The change noted in series I was again one of inanition since animals of the paired feeding group failed to exhibit such differences. It is interesting to consider the maturation followed by atrophy. That this is possibly due to a decrease in male sex hormone is suggested by observations of Moore and Samuels ('31).

Ovary. Female animals in series I coming to autopsy before 28 days showed little difference in the appearance of the sex glands. After this time the ovaries of the controls had more follicles and the follicles looked more mature. The stroma was increased and more compact. No corpora lutea were seen. In contrast the follicles in the deficient animals were smaller and less numerous. The stroma was looser. Follicles undergoing atresia could be found in both. Until the forty-second day, the differences were even more marked since in the control animals large corpora lutea were found while these were absent in the other group. Again, too, the follicles were smaller in the latter animals. From the forty-ninth day on, it was apparent that even in spite of the weight loss occurring in the deficient animals ovulation had taken place in about half of them. However, the corpora lutea were not so large nor so numerous as they were in the controls. In the animals of the paired feeding series autopsied from the forty-ninth to the sixty-seventh day, a difference was noted between the controls and the deficient rats on the low phosphorus diet. The ovaries of the former group were larger and contained mature follicles and corpora lutea. On the other hand the gonads from the latter animals had no corpora lutea and the follicles were smaller and fewer. It is difficult to evaluate these findings. However, in view of the poorer nutrition of the deficient animals as evidenced by the weight (Day and McCollum, '39) it seems not improbable that the factor of inanition was responsible for the differences and that probably the lack of phosphorus did not play an immediate role. This argument is further strengthened by the fact that half the deficient animals of series I revealed evidence of ovulation and the curve of their weight falls between the two groups in the paired feeding experiment.

Uterus. The uteri in series I showed very little difference in the first few weeks. There were occasional glands and a few mitotic figures in the controls. At 28 days, however, there was a distinct difference in size between the two. The epithe-

lial cells of the controls were more columnar and showed vacuoles. The glands were more prominent and the connective tissue stroma was denser. In contrast, the epithelium of the deficient animals was lower and showed little evidence of secretory activity. The glands were inconspicuous. After 28 days the differences were marked and the control uteri grew larger and developed in normal fashion while those of the deficient animals remained atrophic. The uteri from the paired feeding animals of series II showed practically no differences so it appears that the changes found in series I were due to inanition.

Vagina. In the first 2 weeks, no differences could be noted in the vaginal epithelium of the animals of series I. At the twenty-first day, however, there was definite keratinization of the epithelium of the control rats. The vaginas of the phosphorus deficient animals failed to show this change. During the ensuing weeks the epithelium of the controls preserved the same appearance of estrus while similar changes were not noted in the rats on the deficient diet until the forty-ninth day. At this time keratinization of the epithelium had occurred in about half and could be correlated with the state of maturity of the ovary. In the animals of series II autopsied between the forty-ninth and sixty-seventh days, the vaginal changes were inconstant in the rats on diet 16; one showed multilayered epithelium while two did not. The number of females (five) in this series makes it unwise to draw any conclusions as to the possible role played by a deficiency of phosphorus.

Salivary glands. The submaxillary glands were studied in the animals of series I. No changes were detected.

Thyroid gland. The follicles of both groups in series I were about the same size and contained the same amount of pink-staining colloid. However, the epithelial cells of the two groups did differ in that the controls had cuboidal cells while the cells in the deficient animals were a little flatter. There was no change in the stroma of the glands. In the thyroids of the paired feeding series, no differences at all could be detected.

Parathyroid gland. Serial sections were made through the parathyroids in series I. Studies of the volume by the planimeter method as employed by Pappenheimer ('36) were not carried out. However, an attempt was made to gain an estimate of cell size by drawing a number from each gland with

the camera lucida. These tracings were then measured and the average size of the nuclei computed. As far as could be determined, the nuclear size was an index of the size of the cell. By comparison of the average cell diameters of the control and deficient animals it appeared that the controls tended to be a little larger. The differences were ascribed to a difference in the weights of the animals of the two groups.

This study of the parathyroid glands was approached with great interest because of the close relationship of these structures to phosphorus and calcium metabolism. In previous experiments Luce ('23) had found that when young rats were fed on a calcium deficient diet (0.04%) there was an enlargement of the parathyroids due to hyperplasia of their cells. Pappenheimer ('36) found an increase in volume of the parathyroids in rats whose renal tissue had been greatly reduced in amount. That one of the important functions of the parathyroid glands is to regulate the excretion of phosphorus seems well established. This hypothesis has been further strengthened by recent work of Drake, Albright and Castleman ('37) who found that when rabbits were given large quantities of phosphate, their parathyroids were increased in size due to hyperplasia of the cells. On the basis of this, it was reasonable to expect the glands of our animals to be decreased in size since the renal output of phosphorus was extremely low as shown by metabolic studies (Day and McCollum, '39). It is possible that the presence of vitamin D in the diet prevented any change in the parathyroids since a deficiency of this nutrient may be essential in the production of parathyroid hyperplasia (Wilder and Howell, '36). Then, too, since our experiments lasted only 9 weeks, one must consider the possibility that differences would not manifest themselves in this interval.

Muscular system. Sections of striated and smooth muscle from animals of series I showed no lesions.

Bone-marrow. Sections of the medullary cavities of the long bones were studied. In these no change in hematopoietic activity could be demonstrated. As was pointed out above, there was an equal amount of iron pigment in the spleens of

the two groups and there was no indication of excess blood destruction as evidenced by absence of iron pigment in the kidneys of the deficient animals. No blood counts or haemoglobin determinations were performed on these animals. In the recent report of Schneider and Steenbock ('39) no evidence of blood destruction could be detected in acute phosphorus deficiency.

Nervous system. Several sections of the brain and spinal cord of animals of series I dying from the seventh week on were studied. No lesions were found in the tissues of the phosphorus deficient animals. The inability to use the hind limbs was doubtless not due to an anatomical lesion in the cord but to muscular weakness.

Hypophysis. Mann's stain was used to differentiate the various types of cells. No differences could be detected between the size of the cells and the relative numbers of chromophobes and eosinophiles in the animals of series II. The basophiles were scanty in both groups. When those glands were compared with those of the animals of series I there was a detectable atrophy of the cells of the glands of the paired feeding animals. This apparently was similar to the change described by Jackson ('25) and more recently by Werner ('39). The latter author has pointed out that the gonadotrophic function of the hypophysis fails during chronic inanition in the rat and this doubtless accounts for the changes noted in the testes and ovaries of these animals.

Skin. Sections of the dorsal integument were made in animals of series I. In the deficient animals the epidermis was not much different from that of the controls with the exception that there was more keratinization of the epidermis of the latter rats. Little difference was noted in the animals on the paired feeding regime. The corium of the normal animals in series I was definitely thicker than that of the phosphorus low rats but in the animals of series II no marked change could be noted; nor were there any changes in the hair follicles of the rats of this group. The follicles of the normal rats of series I were distinctly larger than those of the deficient animals.

SUMMARY AND CONCLUSIONS

Detailed microscopic studies have been made on tissues from rats restricted to a diet extremely low in phosphorus (0.017%) but adequate in all other respects. Marked skeletal

rarefaction occurs with accompanying rickets. When the factor of inanition is excluded by paired feeding no other definite differences can be detected. The thymus gland and the reproductive system showed the effect of inanition most strongly. The results emphasize the importance of paired feeding in experiments of this nature.

The technical work was performed by Miss Miriam C. Reed.

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PLATE 1

EXPLANATION OF FIGURES

Photographs by Mr. Milton Kougl

1 Low power photomicrograph of proximal end of tibia of rat on P-low diet for 14 days. The ricketic change is indicated by the increase in mature cartilage cells and excessive osteoid.

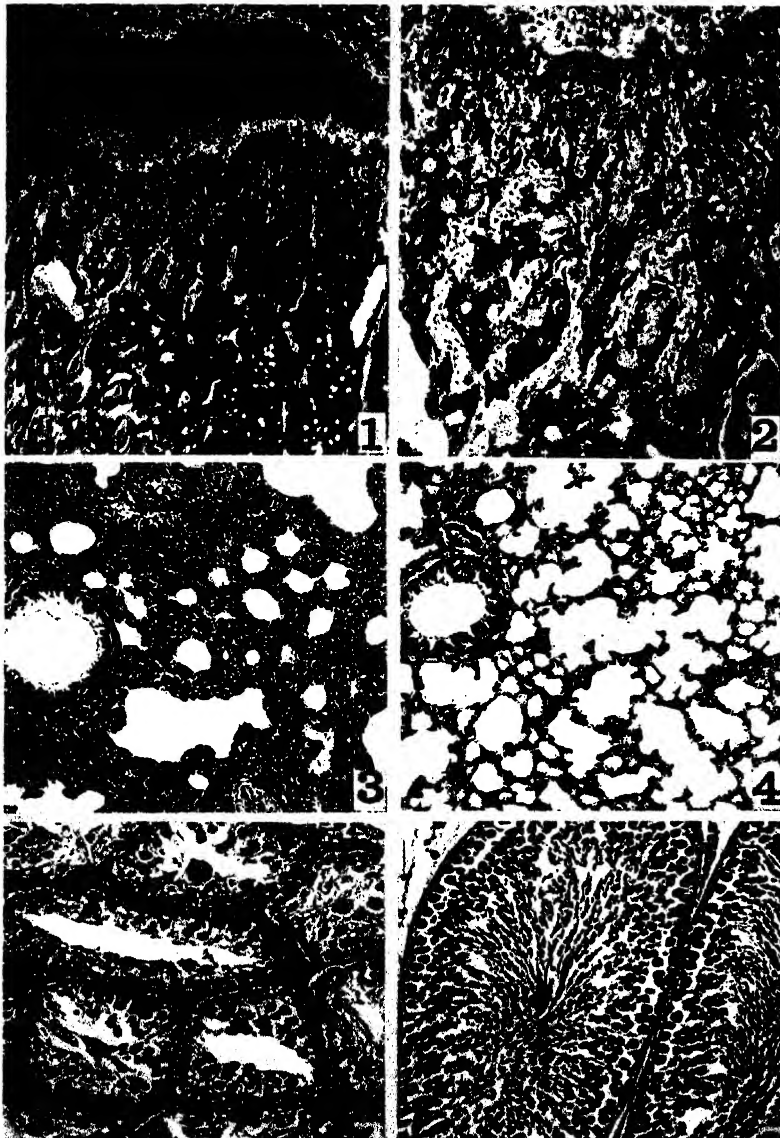
2 Higher magnification of figure 1.

3 Photomicrograph of lung tissue from rat on P-low diet for 49 days. This shows the extreme collapse of most of the alveoli as compared with figure 4.

4 Photomicrograph (same magnification as figure 3) to show normal lung from an ad libitum fed control rat. The alveoli are expanded in contrast to the atelectasis in figure 3.

5 Photomicrograph of testis from animal on P-low diet 53 days. No spermatogenesis is taking place. Note the "giant cells" seen in inanition.

6 Photomicrograph (same magnification as figure 5) of testis from control ad libitum fed animal. The tubules are larger, the cell layers deeper and spermatogenesis is taking place.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FURTHER EVIDENCE OF THE MODE OF ACTION OF VITAMIN D

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Although it is a long since established fact that vitamin D influences the retention of calcium and phosphorus in the animal body, very little is known about the means by which this is accomplished. The theory of Harris and his co-workers ('31 and '32) that the chief function of vitamin D is to increase the net absorption of calcium and/or phosphorus from the gut has found some support in the more recent work of Nicolaysen ('37) and Dols et al. ('37, '38). These later workers however believe the effect on phosphorus absorption is an indirect consequence of primary interference with the absorption of calcium.

On the other hand, the belief that vitamin D functions in the intermediary metabolism of the bone-forming elements (Brown and Shohl, '30; Light, Miller and Frey, '31; Schneider and Steenbock, '39) has been supported by recent work in which an artificially produced radioactive isotope of phosphorus was used (Cohn and Greenberg, '39; Morgareidge and Manley, '39). The conclusion was reached that vitamin D had no influence on the "entrance of phosphorus into the blood but that the healing of the metaphyses of rachitic bones occurs concomitantly with a significant increase in the content of radioactive phosphorus." It is pointed out that this finding is in "contradistinction to any possible role that vitamin D may play in intestinal absorption."

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In experiments recently reported from this laboratory, ('40) it was shown that mineral oil ingestion interfered with the calcifying action of vitamin D when this vitamin was fed as cod liver oil to young dogs and to rachitic rats. The conclusion was reached that vitamin D was dissolved in and excreted with the non-absorbable mineral oil, thus preventing it from playing its part in the calcification process, whatever that role may be.

The experiments reported herein, which were planned to test further the interference of mineral oil, offer additional evidence of the mode of action of vitamin D. In these experiments, vitamin D was supplied by external irradiation of the rat, not by cod liver oil per os as in the previous study.

EXPERIMENTAL

In the first series of experiments, young rats weighing from 50 to 65 gm. were placed on Steenbock's rickets-producing ration no. 2965. When microscopic examination of the tibia of a representative rat from each litter revealed rachitic lesions (twenty-first day) the remaining rats were divided into two groups matched as carefully as possible as to litter, sex and weight, and were treated similarly except that one group was continued on diet 2965 alone and the other was given the same ration in which 10% mineral oil had been incorporated. Paired rats from each group were then exposed at the same time, in a cage especially constructed for this purpose, to the ultra-violet irradiation of a mercury vapor quartz lamp. Except during the period of irradiation which was timed to the second by means of a stop watch, all of the rats were kept in the dark and protected from natural light.

Daily exposures to the ultra-violet light were made for 5 days and on the sixth day the rats were killed and the degree of induced healing of the rachitic lesions as shown by the "line test" (McCollum et al., '22) recorded and graded according to the system of Bills and associates, '31).

In a second series of experiments, rats weighing from 50 to 65 gm. were placed immediately upon diet 2965 with and

without incorporated mineral oil and daily irradiation of both groups begun at once and continued for 18 days. They were then killed and tested for rickets by the usual method. The rickets-preventive effect of irradiation of the mineral oil-fed and non-mineral oil-fed rats was then compared by noting the degree of rickets induced in the two groups of rats. Data on the curative type of experiment are presented in table 1 and results of the preventive experiment are given in table 2.

TABLE 1

Summarized "line test" findings showing the effect of mineral oil ingestion upon the calcification of rachitic lesions induced by daily exposure to ultra-violet irradiation [1]

IRRADIATION PERIOD	DIET 2965 ALONE		DIET 2965 + 10% MINERAL OIL	
	Number of rats	Average degree of healing [2]	Number of rats	Average degree of healing [2]
seconds				
0	8	0.0	10	0.0
15	3	0.0	3	0.0
30	12	1.2	5	0.0
45	10	2.0	—	—
60	15	2.6	12	0.0
90	—	—	8	0.0
120	6	3.8	14	0.7
150			7	1.0
180			8	2.1
240			2	2.5

[1] Exposed to mercury vapor quartz lamp at a distance of 2 feet for 5 days.
[2] Graded according to the method of Bills et al. ('31).

It is quite apparent from results of both procedures that mineral oil ingestion interfered with the action of vitamin D formed by external irradiation of the rat body. "Line test" findings on the bones showed without exception that healing was absent or less advanced in the mineral oil fed group. Whereas a daily irradiation of 45 seconds was sufficient to induce 2+ healing in rachitic non-oil-fed rats, no healing of the rachitic lesions whatsoever resulted from irradiating their mineral oil-fed litter mates simultaneously and for the same length of time. Only when the exposure period was extended to 180 seconds did healing of the same order result.

On the other hand, the preventive tests showed that rickets was more advanced in the mineral oil group than in their litter mates irradiated from the beginning for the same length of time and under the same conditions. Although the development of rachitic lesions was entirely prevented by a 4-second daily irradiation of the non-oil group, the same length of exposure of the oil-fed rats resulted in severe rickets. Daily exposure for 15 seconds was required to form normal bones in the rats ingesting mineral oil.

TABLE 2

Summarized findings showing the effect of mineral oil ingestion upon the prevention of rickets by daily exposure to ultra-violet irradiation

IRRADIATION PERIOD	DIET 2965 ALONE		DIET 2965—10% MINERAL OIL	
	Number of rats	Degree of rickets after 18 days	Number of rats	Degree of rickets after 18 days
seconds				
0	5	Severe [1]	5	Severe
1	2	Moderate [2]	2	Severe
2	4	Mild or incipient [3]	4	Severe
3	4	Mild or incipient [3]	5	Severe
4	4	Normal bone	5	Severe
5	5	Normal bone	5	Moderate
7	5	Normal bone	5	Moderate
10	2	Normal bone	5	Mild or incipient
15	2	Normal bone	5	Normal bone

[1] Characterized by wide, uncalcified metaphysis.

[2] Characterized by narrower metaphysis.

[3] Characterized by swollen epiphyseal diaphyseal cartilage, but without definite metaphysis.

Thus, the presence of mineral oil in the gut seems to interfere with function of vitamin D whether that vitamin be given as cod liver oil by mouth or synthesized by ultra-violet irradiation of certain sterols in the skin. As mineral oil is probably not absorbed from the intestinal canal and therefore must cause its interference there, these results appear to indicate that the vitamin D formed by irradiation reaches the intestinal canal where at least a part of its role is to increase the absorption of calcium or phosphorus or both.

CONCLUSIONS

Mineral oil ingestion has been shown to interfere with both the healing and prevention of rickets in rats on a high calcium-low phosphorus rickets-producing ration which normally results from ultra-violet irradiation. These findings give further evidence that one role of vitamin D is to increase intestinal absorption of calcium or phosphorus either directly or indirectly.

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PIGEON ANEMIA AS A DEFICIENCY DISEASE

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FIVE TEXT FIGURES AND ONE PLATE (TWO FIGURES)

The pigeon has been used extensively in studies of vitamin B (thiamin) but its requirements for other vitamins have been investigated in only a few laboratories. Williams and Waterman ('28) reported that maintenance of weight by pigeons depends on a previously unrecognized vitamin, designated as vitamin B_3 by Williams and Eddy ('28). These same authors (Williams and Eddy, '31) later expressed lack of confidence in the vitamin B_3 hypothesis, but O'Brien ('34), and Waterman and Ammerman ('35) have provided strong evidence in its support. Carter, Kinnersly, and Peters ('30) reported that the pigeon requires still another unrecognized water-soluble vitamin, which they designated as vitamin B_5.

The original purpose of this investigation was to compare the response of pigeons and rats when they receive the same simplified ration, with the idea of concentrating first on the weight maintenance factors. It soon became apparent though (Hogan, Richardson and Johnson, '37), that many of the experimental pigeons were anemic. Doan, Cunningham and Sabin ('25) reported that fasting for 10 to 18 days produced a weight loss of about 125 gm. and a lowering of the erythrocyte count from 4 to 2.75 million per cubic millimeter. Barlow ('27) observed anemia in pigeons which had been maintained on a diet of polished rice, and expressed the opinion that

this was the result of partial inanition. Barlow and Whitehead ('28) studied pigeon blood in some detail and reported that after protracted fasting, or after the development of rice disease, the erythrocytes ranged between 1 and 3.44 million per cubic millimeter.

The chief object of this paper is (1) to describe the technique used to produce the anemia and (2) to present evidence on the nature of the deficiency.

EXPERIMENTAL

Wild pigeons were trapped and used as experimental animals. They were quartered in groups of about six, in wire cages 2 feet square and 14 inches high, with screen floors of 1 inch mesh. Water and food were available at all times but the amounts consumed were not recorded. After the pigeons reached a constant weight, referred to hereafter as normal weight, they were transferred to the experimental ration, no. 1669, composed of casein 20, sucrose 71, salt mixture (Osborne and Mendel, '19) 4, cellulose 3, and cod liver oil 2% respectively.

As was mentioned in the introduction, our original intention was to study the weight maintenance factors. After these studies had been under way some 10 or 12 weeks though, it was observed that some of the pigeons had become pale about the iris. Red cell counts showed that the number of erythrocytes was much lower in all these birds than in those which received the stock ration, and an attempt was made to determine whether the condition is reproducible.

During these preliminary observations, and as is still our custom, the pigeons were first depleted of vitamin B_1 by supplying them with ration 1669 alone. On the average the pigeons lose about 30% of their initial weights by the end of the fourth week, and about 25% develop polyneuritis. Since many will die without developing typical polyneuritis, vitamin B_1, or a combination of supplements, was supplied when the weight had dropped to 70% of the normal level, even if the typical symptoms of a vitamin B_1 deficiency did not appear.

The weekly intervals indicated in the table, graphs, and text are counted from the date the pigeons were first given the experimental ration, no. 1669.

The development of anemia. The anemic condition was first observed in pigeons which were receiving a water extract of yeast that had been irradiated by the method of Richardson and Hogan ('36). At this time none of the water-soluble vitamins required by the pigeon was available in crystalline form, so our early studies were carried out with a variety of more or less crude preparations. Later, when crystalline thiamin and riboflavin became available the observations were repeated, with essentially the same result.

In the exploratory work now to be described three different vitamin B_1 carriers were used: (1) the irradiated water extract of yeast; (2) Jansen's acid clay adsorbate [1] and (3) a vitamin B_1 concentrate.[2]

The irradiated water extract of yeast alone was supplied in daily doses of 300 mg. or more of dry matter. This quantity heals polyneuritis promptly and the birds almost invariably gain in weight although they never regain normal weight unless supplied with a more complete vitamin supplement. This partial recovery may last from 1 to 5 weeks and is usually followed by a decline. The red blood cell counts may begin to fall within 3 or 4 weeks, but occasionally the number remains normal for 8 or 10 weeks. Of the twenty pigeons that received this supplement thirteen became severely anemic in periods varying from 5 to 16 weeks from the time they were placed on ration 1669. Six became mildly anemic, with blood counts ranging from 2.10 to 2.74 million per cubic millimeter, in periods ranging from 7 to 19 weeks. The other pigeon had a subnormal red cell count, from 2.80 to 3.94 per cubic millimeter, but it died after 13 weeks. It was also observed that as the anemia developed some of the erythrocytes became abnormal in shape. These abnormal cells were counted

[1] Kindly supplied by Dr. Jansen.

[2] Kindly supplied by Merck and Co.

over a considerable period of time, and when available the number is shown on the graphs.

Jansen's acid clay, the International Vitamin B_1 standard for some years, was supplied to seven pigeons, in doses of 100 mg. daily. All of the pigeons that received this supplement required an unusually long time to become anemic, and presumably this standard is contaminated with the antianemic agents.

Crystalline vitamin B_1 was not available when these studies were begun, but we were able to secure a highly potent vitamin B_1 concentrate. Of twenty-five pigeons which received this material nineteen became severely anemic in periods of 8 to 13 weeks, and the other six became mildly anemic. Just before this investigation was concluded thiamin became available, and it was supplied to five pigeons. For experimental use the crystalline vitamin is vastly preferable to the more or less crude preparations previously available but pigeons which receive this supplement do not become anemic much sooner than do those receiving the crude concentrates just mentioned. Typical histories of anemic pigeons which received the various vitamin B_1 supplements mentioned are summarized in figure 1.

Effect of depletion of vitamin B on development of anemia. The routine procedure to produce pigeon anemia, as previously described, was to deprive the birds of all members of the vitamin B complex. In an effort to determine whether the anemia would develop if the pigeons received an adequate vitamin B_1 supply continuously while consuming the basal diet, four pigeons received 600 gamma daily of the B_1 concentrate from the time they were given ration 1669. The time required to produce anemia in the controls was 7 weeks, but the non-depleted group had not become severely anemic in 20 weeks. During this period they lost weight gradually and continuously. A summary of these observations is shown in table 1.

Relation of other recognized water soluble vitamins to the development of anemia. It seemed entirely possible that the

anemia and loss of weight were due to deficiencies of some of the known members of the vitamin B complex, hence those then available, riboflavin and nicotinic acid, were combined with various vitamin B_1 carriers. Wheat germ oil also was

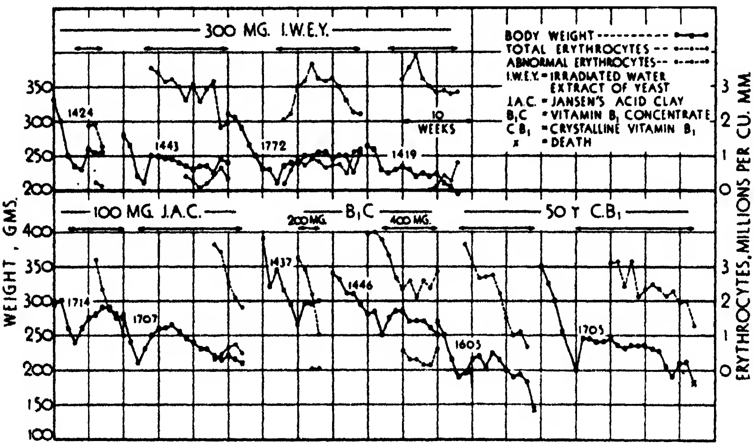

Fig. 1 During the periods indicated by individual arrows the pigeons received vitamin B_1 from various sources of supply. The birds declined in weight and became anemic. Some of the erythrocytes were irregular in outline.

TABLE 1

Average weights and red-cell counts of pigeons not subjected to preliminary depletion of vitamin B_1

EXPERIMENTAL PERIOD	WEIGHT	RED-CELL COUNT
weeks	*gms.*	*millions per cu. mm.*
0	295	3.9 [1]
14	229	2.76
20	208	2.58

[1] The average of all counts made on normal pigeons.

included since it had been shown (Hogan and Richardson, '35) that it heals one type of rat dermatitis. Of the two riboflavin preparations one was crystalline [3], the other was a crude concentrate prepared in the laboratory. The nicotinic acid [4] was

[3] Kindly provided by the Winthrop Chemical Co.

[4] Purchased from the Eastman Kodak Co.

crystalline. In no case did the addition of these substances, or of any combination, have any marked effect on the development of anemia or on the decline in weight. Typical histories of pigeons which received these supplements are shown in figure 2. After these studies were completed other available vitamins were examined but none has been effective.

Anemia not due to fasting. Since the pigeons were subnormal in weight during most of the experimental periods it

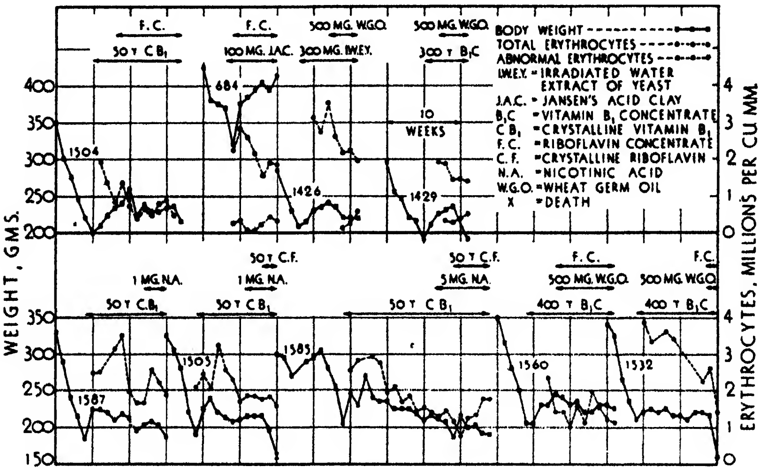

Fig. 2 There was no evidence that the anemia is due to a deficiency of riboflavin, nicotinic acid, or the antidermatitis vitamin of wheat germ oil.

was suggested that the anemia was merely one of the consequences of partial inanition. In our opinion underfeeding does not account for the degree of anemia observed. In the first place many pigeons will make a considerable gain in weight after the administration of vitamin B_1 is begun, although at the same time the red cell counts are declining. Pigeons 1714, 1437, 1446, and 1605 of figure 1, and pigeons 1504 and 684 of figure 2 illustrate this condition. Other examples are shown in the upper half of figure 3, obtained in the course of attempts to find other vitamin concentrates that

would heal the anemia or restore normal weight. These concentrates are not described since none of them was consistently useful. Additional evidence was obtained by using tikitiki, prepared by Wells' method ('21) as a source of vitamin B_1. This material is a potent source of the antianemic agent, but it is deficient in the factors required for weight recovery. It should be pointed out, however, that although in our experience all preparations of tikitiki are active in preventing anemia,

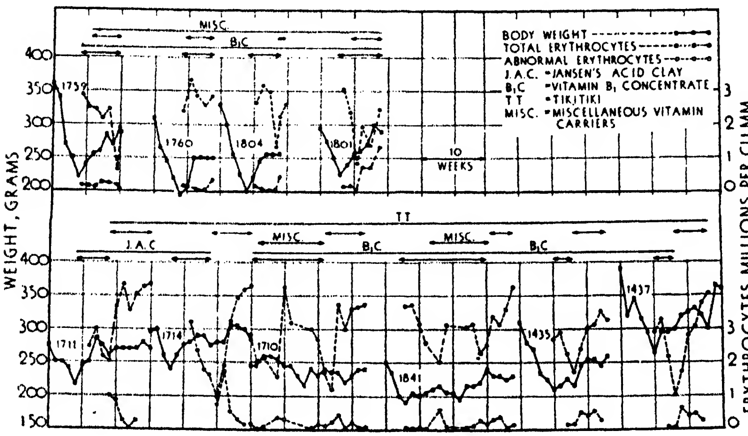

Fig. 3 The upper half of the graph shows that the number of erythrocytes may decline although the pigeons are gaining in weight. Nos. 1759 and 1801 are especially good examples.

The lower half shows that tikitiki permits rapid recovery from anemia, although there may be little or no gain in weight. The periods when the various vitamin carriers were supplied are shown by the individual arrows.

they are variable in their content of the weight recovery factors. On some preparations the gain in weight is negligible, on others the pigeons may recover two-thirds, or more, of the weight lost. Even on these preparations though, the weights will ultimately decline again. Typical responses to tikitiki treatment are shown in the lower half of figure 3.

In order to demonstrate more conclusively that the anemia is not due entirely to a deficient calorie intake, a group of

anemic pigeons was given dried yeast as a curative agent, and at the same time their food intake was restricted so their gain in weight would approximate that of a control group which ate ad libitum, but did not receive any antianemic supplement. As is shown in figure 4 yeast feeding was followed promptly by an increase in the number of erythrocytes, though during this period the red cell counts of the controls steadily declined. It seems clear that the weight maintenance factors are not identical with those that prevent this type of anemia, though they may be interrelated. It is true that Doan, Cunningham, and Sabin ('25) and Barlow ('27) produced a mild anemia by withholding food but their birds were deprived of

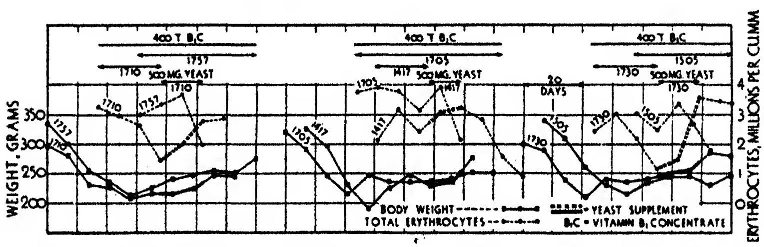

Fig. 4 The number of erythrocytes increased when yeast was consumed, although the food intake of the pigeons was restricted in order to prevent a marked increase in weight. During corresponding periods the number of erythrocytes in the blood of controls continued to decline.

The arrow, which indicates the period during which a vitamin carrier was supplied, bears the same number as the pigeon to which the arrow applies.

vitamins as well as of other nutrients. If pigeons are permitted to consume food ad libitum when yeast is supplied they not only recover from anemia but they also regain normal weight (fig. 5).

Normal erythrocyte count of pigeons. In order to determine the degree of anemia it was necessary to have counts on normal pigeons as a criterion. Ten pigeons were selected at random for these observations, and the counts were made whenever convenient, therefore at irregular intervals. In all, there were forty-six individual red blood-cell counts. The blood was obtained from a wing vein, and diluted 200 times with Tois-

son's solution. The number of red cells per unit volume was rather uniform. The greatest difference in any one individual was 0.78 million per cubic millimeter, and the greatest difference in all counts was 0.86 million. The average was 3.89 million. According to Barlow and Whitehead ('28) the red cells of sixty-eight normal pigeons ranged from 3.78 to 4.52 million per cubic millimeter. In a later publication Barlow ('30) states that the normal erythrocyte count in pigeons is approximately 4 million per cubic millimeter. Kennedy and Climenko ('28-'29) reported an average of 4.3 million for males and 3.6 for females. There is no commonly accepted

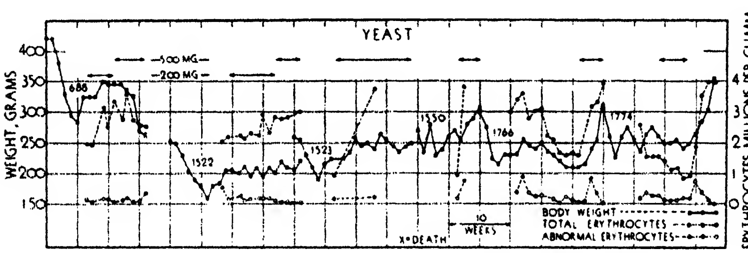

Fig. 5 Pigeons recover from anemia and regain normal weight when supplied with yeast. The response of pigeons 688 and 1522 shows that 200 mg. daily is less than the minimum requirement. The response of the others shows that 500 mg. is enough.

Between the end of the preliminary depletion period and the beginning of yeast feeding various vitamin B_1 carriers were supplied, but these are not shown on the graph.

opinion as to where the dividing line that separates anemic from normal pigeons should be placed but recently we have allowed the number of erythrocytes to fall to between 1.0 to 1.8 million before curative treatment was initiated.

Observations on blood of anemic pigeons. Erythrocytes from a normal and from an anemic pigeon are shown in plate 1. It will be observed that many red cells from the anemic pigeon are abnormal in shape suggesting the sickle-cell anemia occasionally observed in negroes. These deformed cells make their appearance in the blood soon after the number of

erythrocytes begins to decrease, usually in from 5 to 9 weeks after the pigeons have been placed on the experimental ration, no. 1669. The number ranged from 0.10 to 0.30 million per cubic millimeter at the beginning, up to 50% or more of the total number of cells in cases of severe anemia. When the anemic pigeons were given a curative agent, such as tikitiki, the number of deformed cells often increased, and in some cases this increase accounted almost exactly for the total increase in cells. After the first or second week of the curative treatment, however, the number of deformed cells steadily decreased and they usually disappeared entirely from the circulation by the end of the fifth week after the curative treatment was initiated.

As a working hypothesis it is assumed that this type of anemia is due to a deficiency of a previously unrecognized factor, a member of the vitamin B complex. The data do not indicate whether it is a single compound or a mixture.

SUMMARY

1. The procedure followed in producing pigeon anemia is to withhold all members of the vitamin B complex for 4 or 5 weeks, then supply vitamin B_1.

2. The development of anemia was not affected by supplying the pigeons with wheat germ oil, riboflavin, or nicotinic acid.

3. The anemia is not due to partial inanition alone.

4. Some of the erythrocytes of anemic pigeons are abnormal, resembling in some measure the characteristic red corpuscles of sickle-cell anemia.

5. It is suggested as a tentative hypothesis that the anemia is due to a deficiency of a previously unrecognized vitamin.

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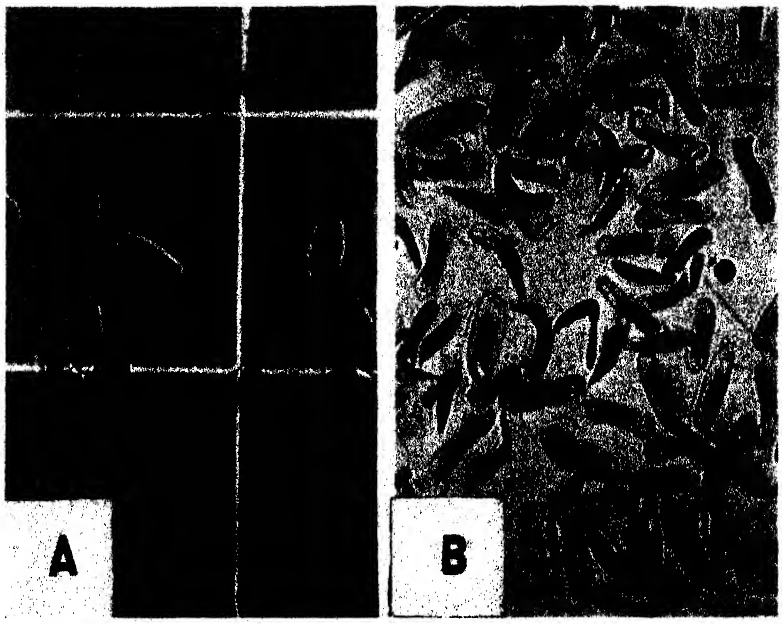

A Erythrocytes of a normal pigeon, 4.08 million per cubic millimeter. Dilution 1 to 200.
B Erythrocytes of an anemic pigeon, 0.74 million per cubic millimeter. Dilution 1 to 20.

THE COMPARATIVE RATE OF ABSORPTION OF SOME NATURAL FATS [1, 2]

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With few exceptions fats have been demonstrated to be almost completely absorbed from the gastrointestinal tract of normal men. In a series of tests on some sixty different types of natural fats by Langworthy ('23) and co-workers, it was found that in practically all cases the coefficient of digestibility exceeded 90%. The chief exceptions noted were in the case of fats having a high melting point such as mutton fat (Langworthy and Holmes, '15), oleostearin (Holmes, '19), and deer fat (Deuel and Holmes, '22), as well as some hydrogenated fats melting from 52° to 60° (Deuel and Holmes, '21) where values as low as 79% were found for the average coefficient of digestibility. McCay and Paul ('38) have noted similar differences in the extent of digestion of the higher melting fats in guinea pigs.

On the other hand, very few studies have been carried out on the rate of absorption of the different fats to ascertain the factors which might alter this property. Steenbock, Irwin and Weber ('36), employing a technic described earlier by Irwin, Steenbock and Templin ('36) reported that lard, hydrogenated cottonseed oil, and butter were absorbed to the extent of about 60% in a 6-hour period after 1.5 cc. of the fat was administered to rats, while linseed oil disappeared

[1] Some of this work was made possible by a research grant from The Best Foods, Inc.

[2] Some of these results were reported at the meeting of the Institute of Nutrition at New Orleans, March 13, 1940.

at a somewhat more rapid rate. On the other hand no sex differences were noted nor were the authors able to correlate the rate of absorption with body weight, surface area, or the length of the intestine.

In the present study the comparative rates of absorption of several fats differing in chemical nature have been investigated. In the first place a fat having a low saponification number (rape seed oil) has been compared with several fats having high saponification values (butter fat and coconut oil). Secondly, a comparison has been made of the rate of absorption of a hydrogenated cottonseed oil with a "wintered" cottonseed oil from which the tristearin had been largely removed. The hydrogenated cottonseed oil was fed in different size doses and to rats which were widely divergent in body weight.

EXPERIMENTAL

The fats were fed by stomach tube to rats previously fasted for 48 hours, the material being measured in tuberculin syringes of 1 or 2 cc. capacity which were connected with the stomach tube. No anesthesia was employed.

The fat could be measured with considerable accuracy by this technic. The results (in milligrams) of successive unselected tests when 1 cc. of the melted fat was discharged into flasks and weighed are as follows: rape seed oil (910, 913, 914), butter fat (908, 907, 910), coconut oil (920, 918, 919), tricaprin (928, 927, 928). When 1.5 cc. quantities were employed, the values for some other fats are as follows: triacetin (1702, 1705, 1713, 1695), tripropionin (1601, 1605, 1605, 1605, 1599) and tributyrin (1540, 1558, 1548, 1554, 1557).

After periods of 3, 4½, or 6 hours the rats were anesthetized with amytal, the intact gastrointestinal tract was removed, and the fats remaining in the gut removed by the procedure of Deuel, Hallman and Quon ('39). In the experiments recorded in tables 5 and 6, the fat was recovered by a modified procedure which was equally as satisfactory. The stomach and entire gut including the caecum and colon were removed intact. In the modified technic, after inserting a blunt needle

into the esophagus, 70 cc. of diethyl ether was flushed through the gastrointestinal tract instead of water and petroleum ether as used in the earlier method. This "washing" was dried with anhydrous Na_2SO_4 overnight; the extract was then filtered into weighed Bailey-Walker fat flasks after which the residue was washed several times with generous quantities of ether. After removal of the ether, the fat remaining in the flask was weighed.

A correction was applied for the recovered fat by subtraction of the quantity of ether-soluble material which was removable from the gastrointestinal tracts of rats fasted for a similar period but fed no fat (table 1). A further correction

TABLE 1

The average lipid content of the gastrointestinal tract of rats previously fasted for 2 days

PROCEDURE AND SOLVENT	NUMBER OF EXPERIMENTS	SEX	BODY WEIGHT	LIPID		
				Maximum	Minimum	Average
			gm.	*mg.*	*mg.*	*mg.*
I, Petroleum ether	20	M	225	23	0	12.5
I, Petroleum ether	9	F	150	5	1	3.0
II, Diethyl ether	11	M	214	43	9	24.9
II, Diethyl ether	10	M	75	50	16	28.9

is made for the extent of recovery of the fat by these procedures based on the values obtained when known amounts of fat are given and the gastrointestinal tract removed immediately (table 2). The results of the control tests are summarized in table 1.

In order to determine the extent of recovery of fats from the gastrointestinal tract by the use of procedure I (warm water followed by petroleum ether) and procedure II (diethyl ether alone), experiments were carried out in which known quantities of fat were administered by stomach tube to fasted rats followed by the immediate removal of the gastrointestinal tract and the flushing out of their contents with the fluid used. The recovery is expressed as the per cent of the original fat given after correction for the control lipid values of fasted

rats. Although a small variation exists in the quantity of material extractable from the gastrointestinal tract of the fasted control rats when petroleum ether and diethyl ether are used, it is apparent that these differences are concerned with their solvent action on metabolic lipid residues rather than on the triglycerides. These results are summarized in table 2.

The rats used in the tests were obtained from our stock colony. They had previously been fed the stock diet described elsewhere (Butts, Cutler, Hallman and Deuel, '35).

TABLE 2

The recovery of various fats from the gastrointestinal tract of rats killed immediately after administration of the fat by stomach tube

FAT FED	PROCEDURE USED	NUMBER OF EXPERIMENTS	SEX	FAT FED	FAT RECOVERED		
					Total	Corrected	Per cent
				mg.	*mg.*	*mg.*	
Cottonseed oil (wintered)	I	11	M	908	876	864	95.2
	I	15	F	1360	1294	1291	94.9
Cottonseed oil (hydrogenated)	I	13	M	809	764	752	92.6
	I	9	M	2835	2726	2714	95.7
	II	12	M	809	782	757	93.6
Rape-seed oil	I	10	M	912	801	789	86.5
	I	10	M	3008	2723	2711	90.1
Linseed oil	I	6	F	928	888	885	95.3
	II	12	M	928	917	892	96.1

In most cases the animals were 3 to 5 months of age with the exception of those used in the experiments recorded in table 5 where the large males were approximately 7 months of age while the young rats averaged about 6 weeks. The weight of the rats was determined after a 48-hour fast. From this value surface area was calculated by the formula of Lee ('29).

The "wintered" cottonseed oil and the hydrogenated cottonseed fat were commercial products obtained on the open market. The hydrogenated cottonseed oil was fed in its

original form as a homogenous mixture with skimmed milk which contained 0.809 gm. of fat per cubic centimeter. The other fats were obtained from known sources. Table 3 gives the constants of the fats used. All were liquid at body temperature. The constants of the fats studied were within the values usually assigned.

RESULTS

In the first series of tests the rate of absorption of the various fats when fed in amounts of 1 cc. was determined in experiments of 3, 4½ and 6 hours duration. This quantity of fat was used because diarrhea frequently occurred when larger doses were employed. After the administration of the fats, the rats were placed in individual cages and the experiments discarded if any evidence of diarrhea was noted. The summaries of the results are included in table 4.

TABLE 3

The physical and chemical constants of the fats used[1]

TYPE OF FAT	SPECIFIC GRAVITY	SAPONIFICATION NUMBER	IODINE NUMBER
Butter fat	0.908	230	28.2
Hydrogenated cottonseed oil	0.903	197	67.6
Wintered cottonseed oil	0.907	190	93.2
Coconut oil	0.919	249	13.0
Rape-seed oil	0.912	175	87.2

[1] These constants were determined by A. Reifman.

In order to determine whether any correlation exists between the size of the animal and the quantity of fat which it is able to digest, the experiments summarized in table 5 were carried out.

The comparisons in the rate of absorption of the same fat when given in increased amounts to rats of approximately the same size are recorded in table 6.

TABLE 4

The absorption of various fats by rats following the administration of 1 cc.

LENGTH OF PERIOD	SEX	NUMBER OF TESTS	BODY WEIGHT	SURFACE AREA	FAT ABSORBED			NUMBER OF CASES OF DIARRHEA
					Per cent of fat fed	Per 100 gm. per hour [1]	Per 100 sq.cm. per hour [1]	
Hydrogenated cottonseed oil (809 mg.)								
hours			*gm.*	*sq.cm.*				
3	M	10	186	287	47.7	69.6±5.2	44.5±3.3	2
4.5 [2]	M	9	159	262	81.4	95.1±5.6	56.9±3.1	
6 [2]	M	10	162	265	90.4	76.0±3.6	46.1±1.9	
6	F	10	152	254	75.5	67.1±2.9	39.7±1.8	
Butter fat (908 mg.)								
3	M	9	174	273	44.7	80.4±4.8	49.6±2.7	5
4.5	M	10	168	268	63.2	79.3±6.4	43.2±3.2	
6 [2]	M	12	197	297	81.6	65.3±4.6	42.6±2.1	7
3	F	6	160	262	45.7	86.4±8.5	52.6±5.1	12
6	F	5	171	273	74.5	67.4±4.7	41.6±2.1	9
Rape-seed oil (912 mg.)								
3	M	11	204	303	37.5	59.4±5.6	38.5±3.0	9
4.5	M	11	175	277	55.8	65.1±3.7	40.7±2.1	1
6	M	11	195	296	58.2	46.1±3.2	30.0±1.8	1
3	F	5	158	261	32.5	62.5±2.5	37.0±1.5	5
6	F	10	146	250	43.4	44.8±2.9	26.2±1.9	
Wintered cottonseed oil (907 mg.)								
3	M	10	210	310	41.8	59.3±3.9	39.8±2.9	3
4.5	M	9	193	293	69.3	74.2±4.2	47.9±2.3	
6	M	10	175	277	72.4	63.5±3.4	39.7±1.5	
6	F	8	146	249	71.5	72.8±3.3	43.3±1.3	
Coconut oil (919 mg.)								
3	M	8	147	249	34.7	71.4±7.2	42.0±4.5	18
4.5	M	11	158	260	59.5	78.2±3.6	46.7±1.8	14
6	M	7	171	274	76.7	70.0±4.5	43.2±2.0	14

[1] Including the standard error of the mean calculated as follows:

$$S.\ E.\ M = \sqrt{\frac{\Sigma d^2}{n}} \Big/ \sqrt{n}$$

where d is the deviation from the mean and n is the number of observations.

[2] Including experiments where practically complete absorption obtained. These must be considered minimum values.

TABLE 5

The effect of size on the rate of absorption of hydrogenated cottonseed oil by rats during a 3-hour period

GROUP	NUMBER OF EXPERIMENTS	AVERAGE BODY WEIGHT	AVERAGE SURFACE AREA	FAT FED IN MILLIGRAMS		FAT ABSORBED			
				Total	Per 100 sq.cm.	Total	Per cent of fat fed	Per 100 gm. per hour [1]	Per 100 sq.cm. per hour [1]
Male rats									
		gm.	*sq.cm.*			*mg.*		*mg.*	*mg.*
I	23	270	360	1087	300	469	43.2	57.8 ± 2.4	43.3 ± 1.8
II	19	65	153	1101	740	254	23.1	134.5 ± 9.7	56.1 ± 3.4
III	15	74	165	502	300	256	51.0	117.9 ± 7.4	53.8 ± 2.6
Female rats									
IV	9	150	253	989	391	289	29.2	64.2 ± 5.4	38.1 ± 3.2
V	16	64	150	452	300	192	43.5	105.2 ± 5.9	43.7 ± 2.1

[1] Including standard error of mean.

TABLE 6

The effect of the amount of fat fed on the rate of absorption in fasting male rats

GROUP	NUMBER OF EXPERIMENTS	AVERAGE BODY WEIGHT	AVERAGE SURFACE AREA	FAT FED IN MILLIGRAMS		FAT ABSORBED			
				Total	Per 100 sq.cm.	Total	Per cent of fat fed	Per 100 gm. per hour [1]	Per 100 sq.cm. per hour [1]
Hydrogenated cottonseed oil									
		gm.	sq.cm.			mg.		mg.	mg.
VI	15	266	357	486	136	291	60.1	36.8 ± 1.6	27.2 ± 1.1
I	23	270	360	1087	300	469	43.2	57.8 ± 2.4	43.3 ± 1.8
VII	10	198	299	940	300	433	46.1	73.9 ± 6.5	48.4 ± 3.9
VIII	13	203	304	1824	600	401	22.0	65.5 ± 3.9	43.8 ± 2.7
IX	11	204	304	2668	900	514	19.2	84.2 ± 3.4	56.3 ± 2.3
III	15	74	165	502	300	252	50.2	115.7 ± 7.5	50.2 ± 3.1
II	19	65	153	1101	740	240	22.8	127.7 ± 9.8	53.1 ± 3.5
Rape-seed oil									
X	15	191	293	882	300	295	33.4	52.8 ± 5.1	34.0 ± 3.1
XI	8	176	278	2154	900	373	17.3	71.4 ± 8.8	44.7 ± 5.0

[1] Including standard error of mean.

DISCUSSION

The differences in the rate of absorption of the different natural fats investigated are not great in spite of a marked variation in the nature of their fatty acid components. In most cases the averages are between 40 and 55 mg. per 100 sq.cm. per hour.

Rape-seed oil consistently seemed to be absorbed most slowly. This is noted in the 3-hour tests although in the experiments continued for 6 hours the variations are extremely pronounced. At the latter period the rates were 30.0 and 26.2 mg. per 100 sq.cm. per hour for the animals receiving rape-seed oil. It seems possible that the rate of hydrolysis of the longer fatty acids in rape-seed oil or their absorption may be even slower than these results indicate. The higher values during the shorter periods (although still the lowest in the 3-hour period) may result from the normal rate of digestion and absorption of the component palmitic, stearic and oleic acids which are in the molecule. After the disappearance of these, the rate falls markedly because of the inability of the higher chain triglycerides to be as readily utilized.

In the experiments with individual fats, it was our experience that large rats absorbed greater quantities during a given interval than small animals. Since this is the case, the rate of absorption of the fat (as expressed in per cent of the administered fat which disappeared) would vary with the size of the rats employed in the tests. If the animals used in comparison of various fats were of approximately uniform weight (as in the experiments of Steenbock et al.), then comparison on such a basis would be valid; on the other hand, if a wide variation in the average weight obtained, such a method of comparison might be open to serious errors. In order to determine whether the rate of absorption might be correlated with body weight or with surface area, studies were made on the rate of absorption in rats of widely varying size. When hydrogenated cottonseed oil was administered in a dose of approximately 1100 mg. to large male rats (group

I), 469 mg. or 43.2% of the total was absorbed; when the same amount was given to small male rats (group II), only 254 mg. or 23.1% of the total fat administered disappeared in a similar interval. Although the results noted when the comparison is made on the basis of body weight (57.8 mg. per 100 gm. per hour for the large rats and 134.5 mg. for the small ones) are equally as variable, the values show much greater uniformity when based on surface area (43.3 and 56.1 mg. per 100 sq.cm. per hour for the large and small rats respectively). These values become practically identical if allowance is made for differences in metabolic activity of the larger and smaller animals. According to Horst, Mendel and Benedict ('34), the basal metabolism for male rats 7 months in age amounts to 755 Calories per square meter per day while that of rats 7 weeks old is 923 Calories. When the rates of absorption of the small rats (whose experiments are recorded in table 5) are multiplied by a correction figure of 0.818 (obtained by dividing 755 by 923), the corrected rate of absorption of the small rats (groups II and III) becomes 45.9 and 44.0 mg. respectively compared with the level of 43.3 for the large animals.

When the doses of the fat administered to the large and small rats were uniform on the surface area basis (i.e., 300 mg. per 100 sq.cm.), then the same quantitative differences in amounts absorbed are still maintained as in groups I and II, but the per cents absorbed of the original quantity fed more nearly approximate each other (43.2% in the large rats (group I), and 51.0% in small rats (group III)). The uniformity in absorption expressed on the surface area is equally as satisfactory, the corrected values being 43.3 and 44.0 mg. per 100 sq.cm. per hour respectively. Similar results are to be noted in the total amount of fat digested by large and small female rats when fed at the level of 300 mg. per 100 sq.cm., but the absorption calculated on the basis of surface area is uniform (38.1 and 43.7 mg. per 100 sq.cm. per hour for the large and small rats respectively).

The per cent of fat absorbed also is inversely proportional to the quantity of fat administered. These results are also in harmony with the report of Steenbock, Irwin and Weber. However, the total quantity of fat which disappears apparently does vary to some extent with the dose given. The greatest alteration is noted in group VI (table 6) where the quantity absorbed was only 291 mg. when fed at the low level of 136 mg. per 100 sq.cm. When the dose was increased to 300 mg. per 100 sq.cm., the quantity absorbed by rats of similar size was 469 mg. in a like interval. Practically no variations could be noted in the rate of absorption when the doses varied between 300 and 740 mg. per 100 sq.cm., although the highest rate of absorption of hydrogenated cottonseed oil (group IX) and of rape-seed oil (group XI) was found when the dose was increased to 900 mg. per square centimeters. Although the dosage of the fats in the experiments recorded in table 4 was not on the basis of surface area, the variation in quantity administered when calculated on that basis was small (300 to 400 mg. per square centimeter) because of the uniformity in size of the rats. The variations in the absorption rate are not to be attributed to different amounts fed.

SUMMARY

A procedure for recovery of fat from the gastrointestinal tract is described in which the average recovery for various fats in eight of the nine series of tests exceeds 90%.

When the same quantities of hydrogenated cottonseed oil were administered to rats of widely varying weights, the amount absorbed is considerably greater in the large rats. However, most uniform results are obtained when the comparison of absorption is made on the basis of body surface area.

In rats of similar size, the quantity of fat absorbed increases somewhat when increased amounts are fed.

No consistent differences in the rate of absorption of hydrogenated cottonseed oil, "wintered" cottonseed oil, butter

fat, or coconut oil were noted. In most cases the rate of absorption lay between 40 and 50 mg. per 100 sq.cm. per hour in experiments of 3, 4½ and 6 hours duration. However, the absorption of rape-seed oil was somewhat slower, the maximum differences being noted in the 6-hour tests where values of 30.0 and 26.2 mg. were obtained on groups of male and female rats respectively.

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THE RATE OF ABSORPTION OF SYNTHETIC TRIGLYCERIDES IN THE RAT [1, 2]

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The physiological behavior of the triglycerides of the lower fatty acids differs in certain respects from that of the higher triglycerides such as tripalmitin and tristearin. In the first place the fatty acids formed on hydrolysis of the lower triglycerides (up to triheptylin) are to some extent water soluble although the latter two acids (caproic and heptylic) are only slightly so (0.4 gm. and 0.24 gm. per 100 cc.; Hodgman, '37). Under such conditions the presence of bile salts is probably not necessary for their absorption. Moreover, there is considerable evidence that no fats lower than tricaprylin can be deposited as such in the tissues even when fed in fairly large doses (Eckstein, '29 a, '29 b). Lastly, triacetin, tripropionin, tributyrin, and trivalerin all have higher specific gravities than water. Although there is no concrete evidence that this may alter their physiological behavior, we have found that extraction procedures suitable for recovery of the fats having a specific gravity less than one are unsatisfactory when applied to these heavier fats.

The lower synthetic triglycerides apparently are readily digested although no studies are available as to their relative rates in vivo when compared with the natural fats or when compared with each other. Weinstein and Wynne ('36) showed that they were readily attacked by pancreatic lipase in vitro.

[1] Some of this work was assisted by a research grant from The Best Foods, Inc.

[2] These data were presented before the Institute of Nutrition at the meeting in New Orleans.

Tripropionin was hydrolyzed most rapidly followed in order by tributyrin, tricaproin, triacetin, and trivalerin. Using a different procedure, Balls, Matlack and Tucker ('37) found that temperature had a marked effect on the rate of hydrolysis of the lower triglycerides. At 0°C. there was a progressive rise in the speed of hydrolysis with the lengthening of the chain which reached a maximum with triheptylin. At this temperature the hydrolysis of the glycerides above tricaprin was practically blank even after a period of 144 hours. On the other hand at 40°C. the maximum speed of hydrolysis occurred with trilaurin and complete hydrolysis was noted within a 3-hour period while the shorter chain triglycerides were more slowly broken down, the minimum value of 28.4% being obtained for triacetin. The only irregularity in the progressive decrease in rate of hydrolysis with the varying length of the carbon chain under the latter conditions was with respect to triisovalerin which was more slowly hydrolyzed than any fat except triacetin. Deuel, Butts, Blunden, Cutler and Knott ('37) also demonstrated indirectly the differences in metabolism of the synthetic fats with fatty acids of eight carbons or less when compared with the natural fats. Since the former could not be stored (possibly also could not be resynthesized in the intestinal mucosa), the glycerol from them is available for immediate conversion to liver glycogen. With the natural fats which contained exclusively fatty acids capable of deposition in the tissues as neutral fat, no glycerol was available for such conversion to carbohydrate. As might be expected, the triglycerides composed of odd-chain fatty acids (tripropionin, trivalerin, and triheptylin) gave rise to greater quantities of liver glycogen than those composed of even-chain fatty acids because the fatty acid portion of their molecules was also glycogenic.

In view of these variations in the in vitro studies, it seemed of importance to ascertain whether similar differences might be noted when the absorptions were compared in vivo. In the present study the extent of digestion and absorption of the synthetic triglycerides from triacetin to tricaprylin as well

as triisovalerin and trilaurin has been studied in rats over a 3-hour period when they were fed in doses equivalent to 300 mg. per 100 sq.cm. of surface area.

METHODS

The rate of absorption of the synthetic triglycerides has been determined by the procedure described earlier (Deuel, Hallman and Leonard, '40). Since some of the fats had specific gravities greater than unity, the earlier procedure which involved their extraction by petroleum ether from a water extract could not be employed. However, by extraction of the

TABLE 1

The recovery of various synthetic fats from the gastrointestinal tracts of rats killed immediately after administration of the fat

FAT FED	NO. OF EXPTS.	AVERAGE WEIGHT	FAT GIVEN	FAT RECOVERED		
				Total	Corrected for blank	Per cent [1]
		gm.	*mg.*	*mg.*	*mg.*	
Triacetin	12	220	1144	1011	987	86.3 ± 0.9
Tripropionin	12	192	1072	1002	990	92.2 ± 0.5
Tributyrin	12	168	1040	991	963	92.7 ± 0.5
Trivalerin	12	228	1020	912	887	87.2 ± 0.7
Triisovalerin	10	213	1002	980	955	95.4 ± 0.2
Tricaproin	11	234	968	899	874	90.6 ± 0.9
Triheptylin	13	223	960	906	880	91.9 ± 0.4
Tricaprylin	9	233	914	886	861	94.2 ± 0.6
Tricaprin	9	176	696 [2]	695	670	96.5 ± 0.4
Trilaurin	11	209	918	909	884	96.1 ± 0.5

[1] Including standard error of mean.
[2] Only 0.75 cc. administered.

gastrointestinal tract with diethyl ether alone without water, the recovery of the fat could be accomplished satisfactorily. The results when 1 cc. quantities of the fats were given by stomach tube and the gastrointestinal tracts immediately removed and flushed with ether are recorded in table 1.

The fats used were obtained from the Eastman Kodak Co.[3] Their purity was established by the determination of the

[3] One of the samples of trilaurin used was kindly furnished us by Dr. Samuel Lepkovsky.

saponification numbers. Other experimental procedures used were similar to those employed in the earlier work (Deuel, Hallman and Leonard, '40).

RESULTS

The results of the various experiments are summarized in table 2.

TABLE 2

The absorption of various synthetic fats from the gastrointestinal tract of fasting male rats during a 3-hour period

FAT FED	NO. OF EXPTS.	BODY WEIGHT	SURFACE AREA	ABSORPTION IN MG. PER HR.[1]	
				Per 100 gm.	Per 100 sq.cm.
		gm.	*sq.cm.*		
Triacetin	12	205	305	102.3±2.9	68.1±1.4
Tripropionin	12	162	264	51.6±3.4	31.4±2.1
Tributyrin	10	190	291	102.3±6.3	65.0±2.5
Trivalerin	9	190	291	50.5±2.9	32.9±2.3
Triisovalerin	6	194	295	69.6±3.3	45.7±2.5
Tricaproin	9	192	292	83.8±2.3	54.5±1.5
Triheptylin	10	207	306	42.0±2.6	28.0±1.6
Tricaprylin	8	226	324	66.5±6.2	45.9±4.1
Trilaurin[2]	5	230	328	31.5	21.9

[1] Including standard error of mean.

[2] No statistical treatment.

DISCUSSION

The neutral fats composed of the lower fatty acids having an even number of carbon atoms are readily broken down and absorbed in the rat. Triacetin and tributyrin, having rates of 68 and 65 mg. per 100 sq.cm. per hour, are more rapidly absorbed than any of the natural fats investigated. Tricaproin is somewhat slower while tricaprylin is absorbed at a level which compares with most of the natural fats.

No successful experiments were completed with tricaprin because in each of twenty experiments a violent diarrhea ensued. The slow absorption of trilaurin is probably to be traced to its high melting point. The fat solidified in a hard mass in the stomach of the rats. Had it been dissolved in a fat liquid at body temperature, the action of the pancreatic

lipase would probably have been facilitated with the result that the absorption would have compared favorably with that of the natural fats.

The fats having fatty acids with an uneven number of carbon atoms were absorbed at 50% of the rate of the corresponding even-chain fats. Thus, the rates obtained for tripropionin, trivalerin and triheptylin of 31, 33 and 28 mg. per 100 sq.cm. per hour respectively are smaller than those for any of the natural or synthetic fats studied for 3-hour periods.

The results yielded by the in vivo studies differ from those obtained by in vitro digestion of similar fats with pancreatic lipase. Not only were no differences observed in the latter tests between odd and even-chain fats but it was also noted that triacetin was least readily broken down. In the present (in vivo) experiments just the opposite results were noted, i.e., that triacetin was the most readily digested of any of the fats studied. Balls, Matlack and Tucker noted that tri-isovalerin was one of the most slowly digested triglycerides; in the present series this interesting fat was digested fairly rapidly at a rate similar to that of tributyrin or tricaproin rather than that of trivalerin.

It is possible that the rate of in vivo hydrolysis is similar to that observed in vitro, but that the differences noted between the results obtained in the two types of experiments may be related to variations in the rate of absorption of the acids resulting from the digestion. As far as the authors are aware, no comparative studies on the rate of the absorption of the short-chain fatty acids have been made although the ready glycogen formation after propionic, valeric and heptylic acids (Deuel, Butts, Hallman and Cutler, '35) would seem to indicate that the absorption is a fairly rapid one. Experiments are under way to compare the rate of absorption of the short-chain fatty acids.

SUMMARY

Triacetin and tributyrin were the most rapidly absorbed fats of any natural or synthetic fats studied; tricaproin and

tricaprylin disappeared somewhat more slowly from the alimentary tract of the rat. Trilaurin was only slowly removed from the gastrointestinal tract presumably because of its high melting point which caused its solidification.

The neutral fats composed of odd-chain fatty acids (tripropionin, trivalerin, and triheptylin) were absorbed at a rate of 50% or less of that characteristic of the corresponding even-chain fats. Triisovalerin was absorbed at a rate which compared with the even-chain fats.

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THE MINIMUM REQUIREMENT OF RIBOFLAVIN FOR THE GROWING PIG [1]

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TWO FIGURES

(Received for publication May 6, 1940)

INTRODUCTION

The necessity of riboflavin in the nutrition of the pig was demonstrated by Hughes ('39). The present investigation includes two experiments conducted to determine the minimum requirement of riboflavin for growing swine; the first was started in November, 1938, and the second concluded in August, 1939.

EXPERIMENTAL

In the summer of 1938, synthetic riboflavin was available and an effort to determine the minimum requirement of riboflavin was begun that fall.

The basal diet consisted of cane sugar 79%, casein (purified) 15%, lard 1%, rice bran filtrate 2%, salt mixture 3%, cod liver oil 10 cc. per pig weekly, thiamin 4 mg. and nicotinic acid 15 mg. per 100 pounds of pig daily. The cod liver oil was given by mouth once each week, while the thiamin and nicotinic acid were dissolved in distilled water and given to each pig by mouth twice weekly.

The casein was purified by the method used previously (Hughes, '39). The rice bran filtrate was prepared by the method of Lepkovsky and Jukes ('35). The product was

[1] The experimental work reported in this paper became cooperative with the United States Bureau of Animal Industry, July 1, 1938.

treated with sufficient fuller's earth to remove the riboflavin and thiamin.

The sugar, casein, lard (melted before being mixed), rice bran filtrate, and salt mixture were mixed weekly and placed in new receptacles (garbage cans) which were paper lined.[2] The salt mixture was the same as that used previously (Hughes, '38).

Five groups of pigs (weighing about 80 pounds at the beginning of the experiment) with five in each lot were fed the following diets beginning December 12, 1938:

Diet 1—Basal (no riboflavin).
Diet 2—Basal + 1 mg. riboflavin per 100 pounds of pig per day.
Diet 3—Basal + 3 mg. riboflavin per 100 pounds of pig per day.
Diet 4—Basal + 5 mg. riboflavin per 100 pounds of pig per day.
Diet 5—Basal with no lard (1 pound sugar replaced 1 pound of lard) + 4 mg. riboflavin per 100 pounds of pig per day.

The pigs used were uniform in size and age. They were fed and watered in steel troughs. Their quarters were similar in size and shape, having concrete floors and wooden partitions. They had free access to inside and outside pens.

The pigs in all lots gained very slowly; the average per pig in all groups at the end of 38 days was only about 10 pounds each. One pig in the control group (diet 1) vomited 3 days after the experiment began, another was unable to get up at the end of 20 days and died the same day. All the pigs chewed at the fences, and became very thin. At the end of the first 40-day period one pig had died, and the others were either down and unable to rise or were very unsteady on their legs. Two pigs were given 5 mg. of thiamin in a vein of the ear on January 20. It seemed to be of some benefit to one pig, this animal becoming more active, but not to the other. Two days later another pig was given 20 mg. in the ear with no perceptible change. The fourth pig was given 10 cc. of wheat germ

[2] The sugar was secured from the California-Hawaiian Sugar Company; the casein from Golden State Company, Ltd.; the lard from Cudahy and Company; the rice bran filtrate from Vitab Products, Inc.; the thiamin and riboflavin from Merck and Company; the nicotinic acid from the Eastman Kodak Company and the cod liver oil used was Squibb's tested oil.

oil orally on January 29 with no apparent success. These pigs (lot 1—basal) gained an average of 21.3 pounds in 73 days or had an average daily gain of 0.3 pounds.

One pig in lot 2 died on the fifty-fifth day of the experiment and three others were down or had extreme difficulty in getting up. Stiffness, unsteady gaits or trembling were apparent in these pigs throughout the major part of the experiment.

In group 3 the conditions were like those of lot 2. There was one death; the pigs were all unsteady on their feet and two were down and not able to rise.

There was one death in group 4, the same amount of stiffness as in the former groups and unsteady gaits. Three pigs were down and unable to get up.

Group 5 were not fed lard. The pigs behaved like those in the former groups, except that they were more persistent in chewing the fences. One pig was down and another died the last day of the experiment. The results of this experiment were not definite. The pigs fed the basal ration (diet 1) gained more slowly than the rest; however, the pigs in the other lots were not normal.

Because the problem had not been solved another experiment was conducted. It was similar in many respects yet differed in some very important particulars. Increasing the thiamin content of the ration, the addition of wheat germ oil and giving ascorbic acid did not materially change the effects in the early experiment. Believing that we were dealing with a multiple deficiency in the first test, it was logical to increase the rice bran filtrate in the second experiment. The lard in the first test did not materially effect the results so it was left out in the second.

The following rations were fed in the second experiment:

Lot 1b—Basal ration, cane sugar 77%, casein (purified) 15%, rice bran filtrate 5%, salt mixture 3%, cod liver oil 10 cc. per pig weekly, thiamin 6 mg. and nicotinic acid 15 mg. per 100 pounds of pig per day.

Lot 2b—Basal ration + 1 mg. riboflavin per 100 pounds of pig per day.

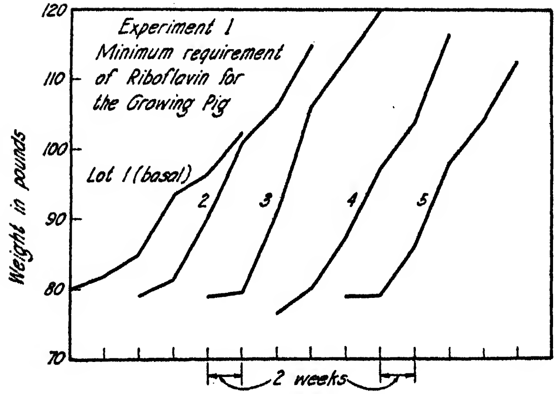

Fig. 1 Growth curves of pigs in experiment 1. The pigs in lot 1 (fed no riboflavin) gained more slowly than those in the other lots. The average age of the pigs at the beginning was about 85 days.

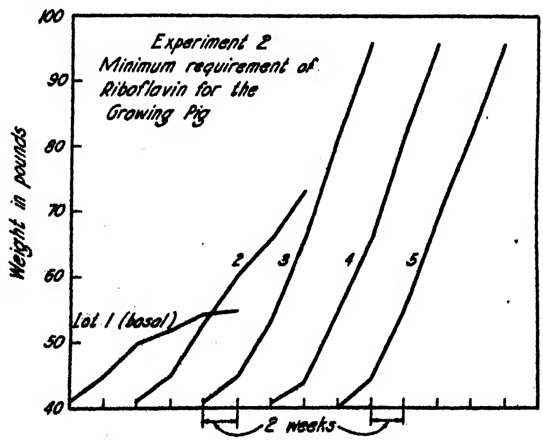

Fig. 2 Pigs on the basal diet gained very slowly; those fed 1 mg. riboflavin daily gained more rapidly (lot 2b); while those in the other groups gained faster than those in group 2b and at the same rate. The average age of the pigs at the beginning of the experiment was about 55 days.

Lot 3b—Basal ration + 3 mg. of riboflavin per 100 pounds of pig per day.
Lot 4b—Basal ration (12 mg. of thiamin instead of 6 mg.) + 3 mg. of riboflavin per 100 pounds of pig per day.
Lot 5b—Basal ration + 6 mg. of riboflavin per 100 pounds of pig per day.

There were five pigs in each group in the second experiment, which were fed in the same pens as those of the first experiment. The thiamin, nicotinic acid, and riboflavin were put in water solution and mixed with the feed each week.

The results of this experiment are graphically represented in figure 2.

The graphs show very slow growth in group 1b, somewhat better growth in lot 2b where 1 mg. of riboflavin was fed. The rate of growth in the other lots was more rapid and practically identical.

DISCUSSION

While the results of the first experiment show clearly the need for riboflavin (see fig. 1, lot 1), there was practically no difference in the growth rates of those in groups 2, 3, 4 and 5. Because there was so much stiffness, staggering gaits, paralysis and some deaths, it was believed that we were probably dealing with a double deficiency in this experiment. In the second experiment the rice bran filtrate was increased to 5% of the diet. This proved to be very beneficial and showed the importance of the filtrate factor, vitamin B_6, or other factors in the rice bran preparation in the nutrition of the pig.

In the second experiment the results are clear cut. The minimum requirement of riboflavin as determined under the conditions of this experiment lies between 1 and 3 mg. of riboflavin (fig. 2) for each 100 pounds of pig per day. The rate of growth for the basal group was extremely slow, and while the growth rate for group 2b, fed 1 mg. of riboflavin, was superior to that of the basal, it was much less than those of groups 3b, 4b and 5b. Increasing the thiamin in group 4b to 12 mg. per 100 pounds was without effect. Doubling the riboflavin given pigs in group 5b over those in groups 3b and 4b was also without effect.

SUMMARY

From the results of these studies, using purified diets, it is concluded that the minimum requirement of riboflavin for the young growing pig lies between 1 and 3 mg. per 100 pounds of pig daily.

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THE MINIMUM REQUIREMENT OF THIAMIN FOR THE GROWING PIG [1]

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ONE FIGURE

(Received for publication May 6, 1940)

INTRODUCTION

Thiamin chloride hydrochloride was given to pigs fed purified diets by Chick et al. ('38), Wintrobe and co-workers ('38) and Hughes ('38). That thiamin is necessary for normal growth and well being was demonstrated by Hughes ('39). In the fall of 1939 it was decided to determine, if possible, the minimum requirement of this factor for the growing pig. Some evidence had been obtained that the requirement was less than 6 mg. per 100 pounds of pig daily (Hughes, '39).

EXPERIMENTAL

The basal diet in this study was the same as that used in determining the minimum requirement of riboflavin for the pig except that thiamin, and not riboflavin, was fed at varying levels. Four groups of pigs weighing between 60 and 65 pounds at the beginning of the experiment were fed the following diets:

Group 1—Basal ration, composed of cane sugar 77%, purified casein 15%, salt mixture 3%, rice bran filtrate 5%, cod liver oil 5 to 10 cc. per head weekly, nicotinic acid 15 mg. and riboflavin 3 mg. per 100 pounds of pig daily.

[1] The experimental work reported in this paper became cooperative with the United States Bureau of Animal Industry, July 1, 1938.

Group 2—Basal + 1 mg. thiamin per 100 pounds of pig daily.
Group 3—Basal + 3 mg. thiamin per 100 pounds of pig daily.
Group 4—Basal + 6 mg. thiamin per 100 pounds of pig daily.

The experiment began December 12, 1939, and continued into February, 1940. There were five vigorous uniform pigs in each lot. The cod liver oil was given by mouth each week while the riboflavin, nicotinic acid and thiamin were put in water solution and mixed with the feed at the beginning of

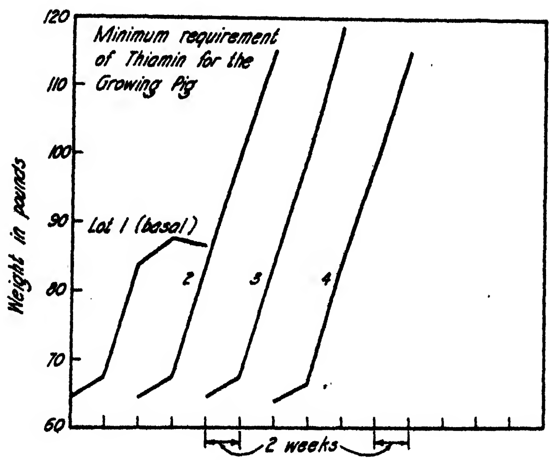

Fig. 1 Growth rates of growing pigs fed varying amounts of thiamin chloride. The average initial age of the pigs was about 65 days.

each week. A week's supply of each ration was mixed on the day the pigs were weighed and it was stored in paper lined, covered cans in a cool place and fed twice daily.

RESULTS AND DISCUSSION

Graphic representation of the results is shown in figure 1. All the pigs grew slowly the first week of the experiment. This was probably due to a change from a barley supplemented normal herd ration to a purified sugar supplemented diet. At the end of the fourth week those on the basal diet (without thiamin) began to exhibit a decline in their rate of

gain and their appetites were poor. At the end of the eighth week they were losing weight. To see if this was caused by a lack of thiamin, 1 mg. of thiamin was added to the diet of this group per 100 pounds of pig daily with the result that these animals gained 9 pounds each during the first week following the addition of this factor.

The rates of gain of the pigs in groups 2, 3 and 4 are very similar. The pigs in group 3 gained slightly faster than those in lots 2 and 4. There was one pig in this group which gained considerably faster than others in the experiment. One pig in each group was killed at the end of the eighth week of the experiment and a sample of the ham muscle of each was assayed for thiamin storage as a further check. From the growth rates and under the conditions of this experiment (fig. 1) it appears that the minimum requirement of thiamin for the growing pig is about 1 mg. per 100 pounds of pig daily. The results of the muscle assay (to be published in another paper) substantiate this assumption.

SUMMARY

Using a purified diet and with growth rate and muscle assay as the criteria, the minimum requirement of thiamin chloride hydrochloride appears to be about 1 mg. for 100 pounds of growing pig daily.

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THE CALCIUM AND PHOSPHORUS CONTENT OF CERTAIN VEGETABLES GROWN UNDER KNOWN CONDITIONS OF FERTILIZATION [1]

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(Received for publication May 8, 1940)

Much experimental work has been done regarding the amounts of fertilizer required by plants and the effect of these upon plant growth. Nearly all of the data concern the yield and quantity of the crop produced, rather than the effects upon quality and mineral content. Smith ('35) stresses the desirability of selecting for commercial purposes those strains of vegetables richest in minerals, realizing at the same time, that there may be considerable variation in composition of the same variety grown in different localities. Bishop ('34) has found that soils low in calcium content yield vegetables poor in calcium, and further that the addition of superphosphate increases the quantity of calcium only slightly. The Alabama soil from which Bishop obtained her vegetables was markedly different from the soil at Geneva. A résumé of pertinent literature to that date appears in her paper. More recent data related to this general subject are present in papers by Davidson and LeClerc ('36), Carolus ('38) and Willard and Smith ('38).

[1] The authors wish to acknowledge the assistance and cooperation of Profs. C. B. Sayre and W. D. Enzie of the New York State Agricultural Experiment Station at Geneva, New York.

[2] The data in this paper are taken from a thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Vital Economics of the University of Rochester, June, 1936.

Examples have been given by Hissink ('30) to show that while the proportions of mineral constituents in plants are dependent primarily on the plant itself, they are also somewhat dependent on minerals already in the soil or added as fertilizers. Coleman and Ruprecht ('35) have concluded that so-called complete fertilizers, when used in amounts for optimum crop production, exert little influence on the composition of crops. When heavy applications of fertilizer were used, effects of soil types on the composition of the crop were nullified.

MATERIALS AND METHODS

The object of this investigation has been to determine the effect of varying amounts of nitrogen, phosphorus and potassium, as supplied in commercial fertilizers, upon the moisture, ash, calcium and phosphorus content of certain vegetables and to correlate these data with certain others reported in the literature.

The vegetables examined in this study were corn (Golden Cross Bantam), green string beans (Stringless Refugee), beets (Detroit Dark Red), and cabbage (Glory of Enkuizen). They had been grown in the summer and harvested in early September at the New York State Agricultural Experiment Station at Geneva, New York. The land upon which the vegetables had been grown is part of a level plain located some 2 miles from the station proper, the soil being of the same type. Here plats, separated from one another by grass walks, had been laid out and each plat had received the same fertilizer treatment for a period of 8 years. Thus, soil conditions were uniform, the only variable being the fertilizer treatment. All crops were exposed to the same climatic conditions, so that for a series of observations made in one growing season, variations in temperature and rainfall may be ignored.

The string beans, corn and cabbage were harvested and brought to the laboratory on the same day. The beets, however, had been harvested a few days earlier and were removed from cold storage. Samples of each vegetable were taken from

plants situated in various sections of the plats to insure average and uniform quality. As soon as possible, the edible portion was prepared as for cooking, the beans and beets being washed free from dirt and wiped to remove adherent water. All samples were then passed through a meat grinder to secure a finely divided, fairly homogeneous mixture. Portions were removed at this stage and analyzed for moisture and ash. The

TABLE 1

Fertilizer used in amounts per acre

PLAT	AMOUNT USED	NH_3		P_2O_5		K_2O	
	pounds	*pounds*	*%*	*pounds*	*%*	*pounds*	*%*
A	600	24	4	96	16	24	4
B	300	12	4	48	16	12	4
C	1200	48	4	192	16	48	4
D	300	45	15	90	30	45	15
E	600	—	—	96	16	—	—
F	600	24	4	—	—	—	—
G*	600	24	4	96	16	24	4
H	None	—	—	—	—	—	—
I	600	24	4	96	16	—	—
J	600	—	—	96	16	24	4
K	600	24	4	—	—	24	4
L	600	48	8	96	16	24	4
M	600	24	4	192	32	24	4
N	600	24	4	96	16	48	8
X	150	6	4	24	16	24	4

* In addition this plat received 70 pounds of copper sulphate and 50 pounds of manganese sulphate per acre.

remainder of the ground material was dried and ashed carefully. Calcium and phosphorus in the ashes were determined in triplicate using procedures outlined in Methods of Analysis of the Association of Official Agricultural Chemists, third edition.

RESULTS

The data in table 1 show the amounts of fertilizer used per acre and the content of NH_3, P_2O_5 and K_2O in pounds and percentage. The size of each fertilized plat was $16 \times 76\frac{1}{2}$ feet. The area from which the crops were harvested on each plat

was 12 × 72½ feet which is exactly 1/50 acre. There was a space of 4 feet between plats to prevent fertilizers from adjacent plats being carried across during plowing and cultivation of the fields. Since the plats were considerably smaller than an acre, only the necessary proportion of fertilizer was used. In all cases the nitrogen in the fertilizer was derived half from ammonium sulphate and half from nitrate of soda. The phosphoric acid was in the form of superphosphate and potash in the form of the muriate. The superphosphate contained about 50% calcium sulphate, so that calcium was supplied to each plat receiving phosphorus fertilizer.

Plat H had received no artificial fertilizer treatment during the last 8 years, although cover crops were turned under each year. Plat X, or the check plat, received only a limited amount of fertilizer which was considered to be the minimum maintenance requirement. The best yields of all vegetables were obtained when the soil received the treatment given to plat A.[3] Plat G was given the same amount and kind of mixture as plat A, with the addition of copper and manganese sulphates.

The percentages of moisture, ash, calcium and phosphorus in the original samples are given in table 2. The data may be interpreted more readily by referring to table 3 during the discussion of results. The average moisture content of the corn, beans and beets was somewhat lower than that reported in the literature. The corn and beans seemed succulent and were being harvested for canning purposes. Since the beets had been in cold storage for a few days, it is possible that some water had been lost. Percentages of water content of all of the vegetables varied, fertilization having apparently little effect upon the corn and beans. The majority of fertilized beets contained less moisture than the lot raised on the check plat, the reverse being true for cabbage (table 3).

Beets were richest in ash being followed in order by corn, beans and cabbage. Considerable variation in ash content was observed among the samples of the four vegetables obtained

[3] Personal communication from Dr. C. B. Sayre, New York State Agricultural Experiment Station, Geneva, New York.

TABLE 2

Effect of fertilizers on content of certain minerals in vegetables

	BEETS					CABBAGE					CORN					BEANS				
Plat	H_2O	Ash	Ca	P	Ca:P	H_2O	Ash	Ca	P	Ca:P	H_2O	Ash	Ca	P	Ca:P	H_2O	Ash	Ca	P	Ca:P
	%	%	%	%		%	%	%	%		%	%	%	%		%	%	%	%	
A	82.1	1.14	0.028	0.035	0.80	93.5	0.61	0.052	0.029	1.80	69.9	0.79	0.0096	0.109	0.089	86.8	0.68	0.076	0.028	2.65
B	83.8	1.09	0.023	0.036	0.63	94.1	0.58	0.045	0.019	2.31	68.0	0.78	0.0084	0.114	0.074	86.3	0.79	0.080	0.036	2.24
C	85.6	1.17	0.024	0.047	0.50	93.5	0.59	0.049	0.024	2.06	69.8	0.73	0.0099	0.114	0.087	85.8	0.78	0.081	0.042	1.91
D	82.0	1.23	0.032	0.048	0.66	93.7	0.56	0.046	0.020	2.34	69.8	0.74	0.0052	0.112	0.047	86.5	0.85	0.080	0.040	2.00
E	81.1	1.15	0.031	0.035	0.88	93.7	0.61	0.046	0.027	1.69	66.8	0.76	0.0072	0.126	0.057	84.0	0.86	0.081	0.042	1.95
F	81.2	1.26	0.033	0.033	1.00	93.7	0.63	0.042	0.022	1.89	69.9	0.80	0.0117	0.097	0.120	85.7	0.71	0.077	0.035	2.20
G	82.9	1.16	0.017	0.044	0.38	94.9	0.58	0.043	0.022	2.00	68.1	0.88	0.0081	0.117	0.069	84.5	0.80	0.087	0.039	2.23
H	85.5	1.08	0.023	0.032	0.72	93.4	0.51	0.040	0.016	2.53	68.8	0.84	0.0127	0.116	0.109	84.6	0.75	0.082	0.031	2.64
I	76.2	1.32	0.032	0.059	0.53	92.8	0.66	0.046	0.025	1.85	70.3	0.85	0.0068	0.117	0.058	84.4	0.81	0.079	0.039	2.02
J	81.3	1.16	0.017	0.055	0.30	94.5	0.59	0.039	0.023	1.69	65.9	0.95	0.0137	0.138	0.099	85.4	0.75	0.085	0.035	2.44
K	81.7	1.11	0.027	0.036	0.75	93.3	0.63	0.041	0.022	1.84	67.7	0.83	0.0063	0.110	0.057	83.3	0.83	0.081	0.047	1.74
L	85.1	1.14	0.027	0.045	0.60	92.6	0.61	0.037	0.025	1.45	66.5	0.88	0.0066	0.132	0.050	83.2	0.79	0.078	0.036	2.14
M	85.6	1.13	0.022	0.049	0.45	94.4	0.55	0.043	0.027	1.60	70.9	0.88	0.0095	0.117	0.081	84.5	0.77	0.074	0.034	2.17
N	86.1	1.04	0.013	0.035	0.38	93.2	0.57	0.042	0.029	1.46	68.8	0.90	0.0077	0.138	0.056	83.7	0.85	0.068	0.053	1.29
X	83.4	1.04	0.028	0.024	1.13	93.4	0.62	0.038	0.024	1.59	69.5	0.92	0.0076	0.125	0.061	81.2	1.00	0.097	0.046	2.12
Av.	82.9	1.15	0.025	0.041	0.65	93.6	0.59	0.043	0.024	1.87	68.7	0.84	0.0087	0.119	0.074	84.7	0.80	0.080	0.039	2.12
Sherman			0.028	0.042	0.67			0.046	0.034	1.32			0.006	0.103	0.058			0.055	0.050	1.10
Bridges			0.024	0.037	0.65			0.045	0.026	1.73			0.006	0.103	0.058			0.050	0.051	1.02

from the various plats, but with the possible exception of corn, the fertilized plats yielded crops richer in ash than those grown on unfertilized soil.

There were marked variations in the calcium and phosphorus content of each of the vegetables examined. Our average percentages are different from those given by Sherman ('37), Bridges ('37) and others. The amount of calcium in corn and beans is considerably higher than that reported by Sherman ('37) while that in beets and cabbage is slightly lower. Since we removed the outer leaves of cabbage in preparing it for analysis, the percentage of calcium was probably

TABLE 3 [1]

Summary—effect of fertilizers on ash, calcium and phosphorus

		MOISTURE	ASH EDIBLE PORTION	Ca		P	
				Edible portion	Ash	Edible portion	Ash
Corn samples compared with control—having	More	7	7	1	0	8	9
	Less	7	7	13	14	6	5
Bean samples compared with control—having	More	6	11	3	2	13	14
	Less	8	3	11	12	1	0
Beet samples compared with control—having	More	3	12	9	8	13	12
	Less	11	2	5	6	1	2
Cabbage samples compared with control—having	More	9	14	11	3	14	14
	Less	5	0	3	11	0	0

[1] A total of fifteen samples of each vegetable was analyzed. The number having more or less moisture, ash, calcium and phosphorus than the unfertilized controls are listed above.

reduced, for Cowell ('32) found the outer leaves contained much more calcium than the inner leaves. We found, as did Bishop ('34), that calcium and phosphorus tended to vary in an inverse manner, which caused marked differences in Ca:P ratios. A glance at table 3 reveals that fertilization reduced the calcium content of the edible portion of corn and beans only. Although the calcium content of the edible portion of cabbage is higher with than without fertilization, the reverse is true for the calcium content of the ash. This is due to the variation in the amount of ash present and its calcium content.

Thus, the cabbage raised on the control plat had the lowest ash content of any of the samples, but the percentage of calcium in this ash was higher than the average percentage for fertilized samples. For this reason the total amount of calcium present in the edible portion of cabbage grown on the check plat was low due not to a reduced calcium content of the ash but to the small amount of ash present in the sample. This accounts for the differences between the results for calcium and phosphorus in the edible portion and ash respectively as shown in table 3.

The data in tables 2 and 3 show clearly the effect of fertilizer in increasing the quantity of phosphorus in the ash and edible portion of vegetables. The larger applications of phosphate did not cause correspondingly greater contents of phosphorus in the vegetables. If the percentages of phosphorus found by us are compared with those reported by Sherman ('37), it is seen that with the exception of corn, our values are lower.

TABLE 4[1]

Average percentage deviation of moisture, ash, calcium and phosphorus of all vegetables from those of control plat H

	CORN	BEANS	BEETS	CABBAGE
	%	%	%	%
Moisture	−0.19	+1.00	−3.28	+0.24
Ash	−0.92	+6.98	+6.34	+16.63
Calcium	−32.78	−7.31	−0.49	−6.27
Phosphorus	+4.16	+17.93	+20.26	+29.77

[1] See explanation of computation in text.

The average Ca : P ratios obtained with corn and beets are quite similar to those calculated from data collected by Sherman ('37), while those for beans and cabbage are considerably higher.

The figures presented in table 4 serve as a brief summary of the analytical findings on the ash itself. An example may be given to illustrate the method by which the data in this table are calculated. The percentage of a specific constituent obtained with the vegetable grown on the control plat is used

as a base. Variations from this are either plus or minus. Using values in table 2 for ash in corn, the percentage of ash in the control sample (plat H) is 0.844. In sample from plat A, this value is 0.794 or a difference of —0.050, or 5.92% of 0.844. After all of the values for each constituent had been so calculated, the plus and the minus percentages were grouped and added, the difference obtained and divided by 14—the number of fertilized samples. How application of fertilizer has reduced the calcium and increased the phosphorus of the vegetables is readily seen. This procedure was followed in obtaining data for calcium and phosphorus also, and all values

TABLE 5

Effects of individual types of fertilization upon ash, calcium and phosphorus contents

TYPE OF FERTILIZER	BEETS			CABBAGE			CORN			BEANS		
	Ash	Ca	P	Ash	Ca	P	Ash	Ca	P	Ash	Ca	P
	%	%	%	%	%	%	%	%	%	%	%	%
NPK Aver. 9 samples	1.13	0.024	0.040	0.59	0.044	0.024	0.84	0.0081	0.120	0.81	0.080	0.039
NP	1.32	0.032	0.059	0.66	0.046	0.025	0.85	0.0068	0.117	0.81	0.079	0.039
NK	1.11	0.027	0.036	0.63	0.041	0.022	0.83	0.0063	0.110	0.83	0.081	0.047
N	1.26	0.033	0.033	0.63	0.042	0.022	0.80	0.0117	0.097	0.71	0.077	0.035
PK	1.16	0.017	0.055	0.59	0.039	0.023	0.95	0.0137	0.138	0.75	0.085	0.035
P	1.15	0.031	0.035	0.61	0.046	0.027	0.76	0.0072	0.126	0.86	0.081	0.042
Cont.	1.08	0.023	0.032	0.51	0.040	0.016	0.84	0.0127	0.116	0.75	0.082	0.031

are based upon the percentage composition of ash and not upon edible portion of the vegetable.

If the effects of different types of fertilizers are considered separately, one finds that nine plats received additions of nitrogen, phosphorus and potassium, whereas one each received nitrogen and phosphorus, nitrogen and potassium, nitrogen, phosphorus and potassium, and phosphorus only. The validity of making comparisons between results from single plats with a single type of fertilization and the average result from a series of plats receiving similar fertilizers but in varying amounts may be questioned. We have, however,

grouped the data on this basis, but the findings are not clear cut (table 5). In the first place there is no consistent effect of any one type or kind of fertilization upon ash content. The calcium content of the edible portion of beets and cabbage is higher in the absence of potassium than with it, but the opposite is true for corn and beans. It would seem in certain instances that a base other than potassium may have replaced calcium in some crops raised on fertilized plats. The phosphorus content of the edible portion of beets, cabbage and corn is higher when phosphorus is used than when it is not, but this is not true for beans.

DISCUSSION

Hall ('10), Miller ('31), Sayre et al. ('31) and others have reported that plants utilize less calcium in the presence of abundant available potassium than in its absence. Little attention has been paid to this effect of fertilization on the calcium content of foodstuffs, and with the widespread use of fertilizers in crop production, it seems probable that the amount of calcium may be reduced considerably. Such considerations are of importance in national nutrition. Peterson, Elvehjem and Jamison ('25) determined the calcium and phosphorus present in samples of cabbage raised in several states, the variation in calcium being 0.029 to 0.056% of the edible portion and in phosphorus 0.017 to 0.057%. Our results are well within these ranges. This emphasizes the need of actual analyses of foods in studies of mineral metabolism.

The effect of phosphorus upon the calcium content of plants is a moot question. Newton ('28) believed that plants grown with liberal amounts of phosphate contained an increased amount of calcium, while Bishop ('34) found only a very slight increase in the calcium content of vegetables when superphosphate had been added to the soil. Our data confirm those of Bishop. Several workers, among them Hartwell ('13), Newton ('28), Clemm ('30) and Mitchell ('31) have shown that the use of phosphatic fertilizers leads to an increased

phosphorus content of the plant. This is clearly illustrated by the results in tables 2 and 3 of this paper.

In computing mineral intake, it is important that the food ingested be analyzed, since ash constituents are frequently lost during the cooking process. Further, in studies of mineral metabolism standard tables of analysis should not be relied upon in compiling mineral intake since marked variations occur from crop to crop and variety to variety. Likewise soil, temperature and other climatic conditions influence the mineral composition of plants, emphasizing again the need of chemical analysis to insure proper interpretation of results.

SUMMARY

A study has been made of the effect of various fertilizers upon the moisture, ash, calcium and phosphorus content of sweet corn, string beans, beets and cabbage grown on the same type of soil during the same season.

Corn and beans grown on fertilized plats contain less calcium than those grown on an unfertilized plat while all vegetables examined contained more phosphorus when given fertilizer treatment. We recognize the fact that this conclusion must be limited to apply to this particular soil and the fertilizers used. It is suggested that calcium may be replaced by another base, possibly potassium, supplied by the fertilizer.

Since the calcium and phosphorus contents of the vegetables vary widely and frequently in opposite directions, Ca : P ratios fluctuate between rather wide limits.

The importance of chemical analyses of food in metabolism studies is stressed because foodstuffs grown on different soil types under varying conditions of fertilization, culture, and climatic conditions may exhibit marked differences in composition.

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THE METABOLISM OF CITRIC ACID BY INFANTS [1]

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It has long been known that the salts of the organic acids occurring in fruits and vegetables are, with few exceptions, metabolized to alkaline end-products in the body. The extensive literature on this point has been reviewed by Smith and Orten ('37). Furthermore, the observation has been made that, like natural fruit juices, the ingestion of sodium citrate results in an increased pH of the urine and an augmented excretion of citric acid whereas an equivalent amount of free citric acid has little obvious influence on the composition of the urine (Östberg, '31; Kuyper and Mattill, '33; Boothby and Adams, '34). It has been shown that the alkalinization produced by sodium bicarbonate and by sodium acetate likewise increases the citric acid in the urine from which evidence the conclusion has been reached that the organism has the ability to synthesize citric acid (Schuck, '34a,b; Sherman, Mendel and Smith, '36a).

The distinct metabolism of free citric acid has been emphasized by the almost complete disappearance (insofar as examination of the urine is concerned) of this compound in adult human subjects (Östberg, '31; Kuyper and Mattill, '33; Schuck, '34b), in the dog (Sherman, Mendel and Smith, '36b), in the pig (Woods, '27; v. Fürth, Minnebeck and Edel, '34)

[1] A preliminary report was presented before the American Institute of Nutrition, New Orleans, March, 1940.

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and in the rat (Kuether, Meyer and Smith, '40) after administration by mouth. Gonce and Templeton ('30) examined the urine of four children ranging from 7 to 12 years of age before and after giving 4 gm. of "dehydrated" citric acid per 15 pounds body weight. The results were irregular but in one subject the ingestion of the free acid resulted in a pronounced drop in the concentration of citric acid in the urine. In a study of the factors influencing the retention of calcium, phosphorus and nitrogen of infants who were fed whole milk, Jeans and co-workers ('36) made a comparison of the metabolism of the food citrate on the one hand, and of added citric acid, on the other. With four infants ranging in age from 7 weeks to 26 weeks, 0.088 to 0.206 gm. of citric acid were excreted in the urine when 1.47 to 2.72 gm. per day of food citrate were consumed; when, after adding free citric acid as a curdling agent to the milk, the citric acid intake was increased from 3.16 to 5.02 gm., the output remained at 0.097 to 0.213 gm. These data indicate complete destruction of citric acid when fed as such to this age group.

In their study of the metabolism of orally administered citric acid in the dog, Sherman, Mendel and Smith ('36b) found no increase in fecal citric acid when from 0.5 to 2.0 gm. of the free acid per kilo body weight was given. Langecker ('33) detected no citric acid in the feces of rabbits after feeding either sodium citrate or citric acid. The studies of Kuether, Meyer and Smith ('40) show that the feces of rats contain small amounts of citric acid and that the administration of free citric acid has little, if any, influence on the quantity lost by way of the intestine.

That considerable amounts of citric acid appear in the feces of human subjects was demonstrated by Smith, Bauguess and Barnes ('39) in a study of eleven infants from 3 to 14 months old; they reported analyses showing that from 1.0 to 7.8% of the citrate ingested by this group appeared in the feces. The present study is an extension of their work; it deals with the comparison of the metabolism of food citrate and of free citric acid as determined by the balance method.

EXPERIMENTAL PART

Methods

The subjects of this study were eight infants from 4 to 12 months of age with varying degrees of mild clinical rickets but without other abnormalities. They were given as much as they would voluntarily consume of a formula made up of dried milk, barley flour and *B*-lactose and also canned cereal, soup and apricot-apple sauce. Accurate account of food consumption was kept at all times. During the collection periods (3 days in length and marked by carmine) the babies were maintained on metabolism frames which permitted a complete separation of urine from feces and accurate quantitative collection of both. The data discussed herein are derived from one period during which free citric acid (365 mg./kg. body weight per day), was given by gavage in divided doses and one or two basal periods without added citric acid, for each subject.

The urine was collected in hydrochloric acid and the feces were digested with concentrated hydrochloric acid to a uniform suspension. For the determination of citric acid in the food, feces and urine, the method of Pucher, Sherman and Vickery ('36) was used, the final measurement being made photoelectrically, using a color filter having a maximum transmission at 4250 Å. Approximately 1 gm. samples of the separate food materials were thoroughly stirred with acid (70 cc. water and 3 cc. concentrated sulfuric acid) and extracted on a hot plate; after making to volume and filtering, the determination was made directly on the filtrate in most cases. The reconstituted dried milk was treated with trichloracetic acid and the determination made on the protein-free filtrate. The analyses for the citrate content of the food materials appear in table 1. Inasmuch as it was shown that all of the citric acid of the digested fecal suspension was in the fluid part, samples for analysis were measured directly with a pipette.

DISCUSSION OF RESULTS

It is obvious from the analyses of the food materials shown in table 1 that all of the constituents of the diet contained citric acid but that the dried milk and the canned fruit mixture were particularly rich in this respect. In the course of the experiment the food of the individual infants contributed from 1.091 to 2.502 gm. citric acid per day (see table 2).

In the report of Smith, Bauguess and Barnes ('39) attention was called to the not inconsiderable elimination of citric acid in the feces although it was pointed out that the larger part of the excretion was through the urine. In the present study it is shown again that the feces always contain citric acid in varying proportions of the intake but ordinarily less than that appearing in the urine. The source of this citric acid may be the food, digestive or other body fluids entering the intestine or the activity of the intestinal bacteria. Incubation of known amounts of citric acid with infant feces for 44 hours produced, in our experience, neither destruction nor formation of citric acid. This observation agrees with that of Langecker ('34) who found that the bacteria and enzymes

Table I.
Citric Acid Content of Diet Constituents

Material	Citric Acid
	Per Cent
Barley Flour	0.047
* Milk Powder	1.400-1.625
B-Lactose	0.000-0.006
** Cereal	0.132
** Soup	0.078
** Apricot and Apple Sauce	0.638

* Klim
** Gerber's

in the intestinal tract of the rabbit do not destroy citric acid. In another study (Kuether, Meyer and Smith, '40) it has been shown that incubation of the intestinal contents of the rat with free citric acid for 44 hours results in no detectable destruction or formation of citric acid. In subjects ER, TA, WF and MC the large losses of citric acid in the feces during the period of free citric acid administration are correlated with diarrhea, probably induced by the citric acid itself. The food and possibly the intestinal secretions, thus appear to be responsible for the presence of citric acid in the feces. It is worthy of comment that, in the absence of diarrhea, fecal citric

Table II. CITRIC ACID BALANCE

Subject	Body Wgt.	Citric Acid Intake per 24 hrs			Citric Acid Excretion per 24 hrs			Excretion of Citric Acid in Per Cent of Total Intake			Citric Acid Absorbed in Per Cent of Total Intake	Urine / Absorbed x 100	Urine / Intake x 100
		Food	Extra	Total	Urine	Feces	Total	Urine	Feces	Total			
	KG	GM	GM	GM	GM.	GM	GM						
E.R ♂	6.84	1.694		1694	.168	.126	.294	9.9	7.4	17.3	92 6	10.7	9.9
	5 93	1 174	2.00	3 174	.095	.416	.511	3.0	13.1	16.1	87.0	3.7	8.1
A.K. ♂	8 33	1.909		1909	161	.068	.229	8 4	3 6	12.0	96.4	8 7	8.4
	8 76	2.502		2 502	.165	.079	.244	6 7	3 2	9.9	96 8	6 9	6.6
	8.19	1091	3.00	4 091	.094	.032	.126	2 3	0.8	3.1	99.0	2 3	8.6
F.F ♀	8.23	2.077		2.077	.160	.271	.431	7 7	15 0	20.7	87 0	8 8	7.7
	8.65	1 325		1.325	.128	.093	.221	9.7	7.0	16 7	93.0	10 4	9.6
	7.81	1993	2.70	4.693	.062	.086	.148	1 3	1 8	3 1	98.0	1 3	3 1
T.A. ♂	10.34	2.464		2464	.237	.073	.310	9 6	3.0	12 6	97.0	9.9	9 6
	9.24	2.169	3 30	5 469	.101	.650	.751	1 8	11 9	13.7	88.0	2 1	4 7
J.A. ♂	8.46	2.007		2.007	.230	.066	.296	11 5	3.5	15.0	96.5	11 9	11.4
	7.25	1 654	2 58	4 234	.160	.080	.240	3.8	1.9	5 7	98 0	3 9	9 7
R.P. ♂	7.03	1 328		1.328	.087	.067	.154	6.6	5.0	11.6	95.0	7.0	6.6
	8.05	1 788		1788	.132	.173	.305	7.4	9.7	17 1	90.3	8 2	7.4
	6.87	1.672	2.58	4 232	.042	.340	.382	0.9	8.0	8 9	92 0	1.0	2 5
W.F ♂	8.43	2081		2081	268	.040	.308	12.9	1.9	14.8	98.1	13 1	12.9
	10.57	2.207		2 207	.221	.073	.294	10.0	3.3	13 3	96 7	10.3	10.0
	9.30	1954	3 18	5 134	.072	1.000	1 072	1 4	19.5	20 9	80.0	1 8	3.7
M.C. ♂	8.23	1 760		1760	162	.058	.220	9 2	3.3	12 5	96 7	9 5	9 2
	7.68	1 628	2 78	4 408	.064	1.066	1.130	1.5	24 1	25 6	76.0	2.0	3.9

acid represents a lower proportion of ingested citric acid in the periods when the free acid is fed than in the control periods.

The above observations permit the conclusion that the citric acid which disappears from the intestinal tract is absorbed. From table 2 it can be seen that the proportion of ingested citric acid which is absorbed is high but is decreased in those instances where diarrhea was present. A different picture is presented by the data for citric acid excretion in the urine; here both absolutely and in relation to the quantity ingested, the values for the concentration of citric acid are strikingly

lower during the period when free citric acid was fed than in the control periods when the citric acid was obtained only from the food. A comparison of the proportion of ingested citric acid which was absorbed with the proportion appearing in the urine, provides an index of the completeness of metabolism of this substance. The thirteenth column in table 2 indicates that within the control periods and within the free citric acid-feeding periods, the extent of metabolism of the absorbed citric acid is uniform for the various subjects. However, when one compares the extent of metabolism of the free acid period with that of the control periods, there is a striking difference; the proportion of the absorbed citric acid escaping destruction averaged 9.6 (6.9 to 13.1) % in the control periods and 2.3 (1.0 to 3.9) % in the free acid periods. In the light of the failure to store citric acid in the body under ordinary conditions (Smith and Orten, '37) these data strongly suggest the interpretation that ingested free citric acid is either largely destroyed in the body or transformed to a non-citrate type of compound (see MacKay, Carne and Wick, '40).

A comparison of the amount of citrate ingested in the food with the concentration of urinary citric acid may be taken as an index of the influence of the diet on endogenous citric acid production inasmuch as both the salts of the organic acids occurring in foods and the major foodstuffs themselves, are known to affect citric acid synthesis. Such a comparison, shown in table 2, indicates that in seven of the eight subjects, the ingestion of free citric acid decreases the ratio of urinary to food citric acid, which fact may be interpreted to indicate that not only is the free citric acid metabolized but that, under its influence, the basal production of citric acid as affected by the diet, is inhibited. A similar comparison of the data from like experiments with the rat (Kuether, Meyer and Smith, '40), shows that, whereas the administered free citric acid completely disappears with this species it does not interfere to the same extent with the formation of urinary citric acid as influenced by the diet. The synthesis of citric acid, which can be demonstrated in the rat (Smith and Meyer, '39, Kuether,

Meyer and Smith, '40) is not so striking in the human subjects herein described.

Experimental data are available showing that under certain conditions, the excretion of citric acid by the kidney is suppressed. Thus the administration of hydrochloric acid or of potentially acid substances like calcium chloride and ammonium chloride, is followed by a decrease in citrate concentration in the urine (Östberg, '31; Kuyper and Mattill, '33; Boothby and Adams, '34). Again, Smith and Meyer ('39) have demonstrated that in the rat, the production of endogenous citric acid is greatly diminished when the animal is maintained on a high-protein diet. In the present investigation the failure to increase urinary citric acid apparently is not due to its effect on the acid-base equilibrium for it has been shown by Schuck ('34b) that ingestion of the free acid produces little if any variation of the pH of adult human urine and the same observation has been made in the rat (Kuether, Meyer and Smith, '40).

The present study shows again that the metabolism of free citric acid is not the same as that of food citrate or sodium citrate insofar as can be judged by the excretion of citric acid in the urine. That the metabolism of the free acid exerts a marked effect on that of food citrate is demonstrated by the data. From these balance experiments one can conclude that the fate of food citrates and of free citric acid in the infant is like that in adults and in the other mammals which have thus far been studied.

SUMMARY

Based on the data derived from a series of balance studies with infants, it has been shown that the oral administration of free citric acid results in a marked decrease in the proportion of the ingested citric acid and food citrate appearing in the urine, as compared with that of the control period in which the basal diet is the only source of citric acid. Ordinarily, the feces contain a small amount of citric acid; the citric acid of intestinal contents is uninfluenced by the addition of free citric acid.

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THE UTILIZATION OF CALCIUM IN CARROTS, LETTUCE AND STRING BEANS IN COMPARISON WITH THE CALCIUM IN MILK [1]

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Although it is generally known that the value of a food as a source of calcium in the diet depends upon the results of biological as well as chemical tests, foods are nevertheless evaluated largely on the basis of their contents of calcium. This situation is the result of the incompleteness of available information on the extent to which the calcium of different food materials is utilized in the animal body, and particularly in the human body.

With reference to carrots, the available information has been interpreted in support of quite discordant conclusions. The experiments of McClugage and Mendel ('18) on two dogs have been interpreted to mean that the addition of carrots and spinach to the diet "does not always yield a pronounced advantage to the calcium metabolism. It would, therefore, presumably be an unsafe procedure to use vegetables extensively as a dietary substitute for milk in the nutrition of children." Sherman and Hawley ('22) in experiments on children, concluded that "milk is much superior to vegetables as a source of calcium for the growing child." The vegetables compared with milk were a mixture of carrots, spinach and celery. Edelstein ('32) has also interpreted his metabolism data on children as indicating a decreased retention of

[1] This investigation was aided by the donation of funds by the American Dry Milk Institute to the University of Illinois.

calcium following the addition of carrots or spinach to the diet, consequent upon an impaired intestinal utilization. On the other hand, Rose's ('20) observations on women present no clear distinction between milk and carrots with respect to their values in furnishing calcium to cover the requirement of the adult organism.

These diverse conclusions regarding the value of carrots as a source of dietary calcium seem hardly to be justified from a critical study of the supporting data. The conclusion of McClugage and Mendel was based upon measures of the calcium utilization in the test foods obtained by expressing the negative calcium balances, secured in all experimental periods, as percentages of the calcium intakes. The greater these percentages, the poorer the utilization of dietary calcium was presumed to be. But such a calculation neglects the endogenous loss of calcium from the body. In making a correction for this body loss of calcium, it may be assumed, in conformance with recent evidence, of which the report of Fairbanks and Mitchell ('38) may be cited as an example, that the calcium of spinach is totally unutilizable in the animal body. This being true, the negative calcium balances of the dogs on the spinach diets would be equal to the endogenous calcium losses. These losses amount to 17.6 mg. of calcium per kilogram of body weight for dog A, and 21.6 mg. for dog B, values approximating the average of 18.3 mg. that may be computed from a number of experiments on dogs receiving low-calcium diets (Mitchell and Curzon, '39; see table 2). The differences between the negative calcium balances of the spinach periods and those obtained with the other test foods would measure the replacement of the endogenous calcium losses by the respective dietary forms of calcium. These replacements of calcium expressed as percents of the respective intakes of calcium would measure the utilization of the latter in the adult dog. For milk the percentage utilizations are 52 and 40 for dogs A and B, respectively, and for carrots, 24 and 29. These corrected values still indicate a marked superiority of milk calcium over carrot calcium, but the latter is distinctly

of value in the calcium metabolism of the body, being about 60% as valuable as milk calcium.

The poor showing of a mixture of vegetables including carrots in the calcium economy of the children in the experiments of Sherman and Hawley may be accounted for, to a large extent, by the inclusion of spinach in the mixture. This vegetable possesses the distinction, not only of containing calcium in an unutilizable form, but of probably impairing the utilization of the calcium of foods with which it is fed, because of its excessive content of oxalic acid.

The conclusions of Edelstein do not discriminate between the results with carrots and those with spinach. The addition of the latter food to the basal milk diet almost always induced a marked decrease in calcium retention, while the addition of carrots in two experiments led to discordant results, i.e., a marked decrease in calcium balance in one case, but a slight improvement in balance in the other. Such results justify no definite conclusion.

The experiments of Rose, involving intakes of calcium sufficient in all but one period (a carrot period) to induce slight retentions of calcium, may be unsuited to the detection of small differences between milk and carrots with respect to the dietetic value of their calcium contents. If the adult subjects of these experiments were saturated with respect to calcium, the consumption of calcium in amounts above those required for equilibrium would lead to a wastage of the excess intakes without regard to their inherent biological values. Under these conditions, differences in biological value would be obscured. It seems to be true that, in experiments on adult subjects, the relative values of different sources of calcium can be measured with assurance only if the subjects are in negative balance.

The only experiment concerned with the value of lettuce as a source of dietary calcium is the experiment of Mallon, Johnson and Darby ('33). The results secured were interpreted to mean that the calcium of lettuce was superior in its utilization by healthy women to that of pasteurized whole milk

fed at an approximately equal level of calcium. The foods tested accounted for 93 and 88%, respectively, of the total dietary calcium. But here, also, the calcium intakes were such that positive balances were secured in all periods. The fact that greater positive balances were secured in the first periods with each subject, when the lettuce diets were being consumed, may have been a result of an immediately prior period of inadequate calcium nutrition, rather than of a more utilizable calcium supply in the diet. This experiment again illustrates the difficulty of interpreting calcium metabolism studies on adult subjects in which more dietary calcium is provided than is needed for equilibrium.

The purposes of the experiments to be reported below were to measure the relative value in the nutrition of the growing animal of the calcium in milk and in carrots, lettuce and green string beans, and to determine whether cooking with steam and commercial canning processes modified the nutritive value of the calcium of vegetables. Since in these comparisons, and in the future ones that we plan to undertake, it is more convenient to use commercial skim milk powder [2] than fresh skim milk or fresh whole milk, a comparison of the value as sources of calcium of liquid skim milk and of the commercial powder was also made. This comparison would indicate whether commercial desiccation impairs the value of milk as a source of dietary calcium.

METHODS

The various experiments reported in this paper differed in many minor details of procedure, the nature of which it does not seem profitable to describe, but they were all carried out according to the following general plan.

The rats were taken for experiment at initial weights of about 30 to 50 gm. They were paired, or divided into groups of three (trios), on the basis of initial body weight (taken

[2] This product is now almost universally sold under the name "dry milk solids not over 1.5 pct. fat", and in the discussion below it will be referred to as "dry milk solids."

after a 24 hour fast), sex, and litter membership. Litter mate controls were sacrificed for analysis in order to estimate the initial content of calcium.

The basal diets were of two types. In the early experiments the protein, amounting to about 30% of the basal diet, was provided by dried, ether-extracted whole egg. These diets were fairly high in calcium, containing generally from 0.12 to 0.15%. With so much calcium in the basal diet, the proportion of the total calcium intake provided by the test supplements ranged from 40% up to only 80% depending upon the rate of gain, which determined the amount of supplement given. In the later experiments, the protein was provided by dried, ether-extracted beef round. These diets contained about 0.04% of calcium. The phosphorus contents of all basal diets ranged from 0.25 to 0.35%. The remainder of the basal diets contained in percentages, 10 of sucrose, 10 of lard, 10.5 of dried yeast, 1.5 of a fortified cod liver oil, 4.5 of a calcium- and phosphorus-free salt mixture, otherwise complete, and starch to make up the deficit.

The basal diet was fed in equal amounts to pair mates and trio mates, since the differences in calcium storage produced must be referable solely to the qualitative differences in calcium supply. In most contemporary publications in this field of investigation this precaution is not taken. The tacit assumption is made that the percentage of the calcium intake retained in the body is not influenced either by a variable intake of food or of calcium. This proposition has never been proved and seems inherently improbable. By equating the intake of food and of calcium for comparable animals, the uncertainties in interpreting the results of ad libitum feeding experiments are entirely avoided.

The test supplements were fed in addition to the basal diets in amounts to provide the same quantity of calcium to pair mates or trio mates, but less than that required for maximum calcification. Since maximal calcification involves the production of growth increments in body weight containing about 10 mg. (or somewhat more) of calcium per gram, the calcium

supplements were fed in amounts to provide only from 4 to 7 mg. of calcium per gram of gain, depending upon the concentration of calcium in the test food. With foods low in calcium, less calcium was fed per gram of body weight gain; but even so, enough of the test food had to be given to restrict seriously the intake of basal diet, which should provide most of the nutrients other than calcium. Hence, the basal diet was designed to be liberally provided with protein, minerals other than calcium and phosphorus, and vitamins.

The unequal amounts of supplements given to pair mates or trio mates to provide equal amounts of calcium, would tend to produce differences in body weight gains. Since unequal gains between (or among) otherwise comparable animals, even with equalized intakes of calcium, may modify the retention of calcium (Fairbanks and Mitchell, '36), the gains within pairs and trios were kept equal throughout the feeding period by giving sucrose in concentrated aqueous solution to the laggard rat.

The feeding was continued until the rats in each pair or trio had gained 90 to 100 gm. in body weight, requiring from 6 to 11 weeks, though ordinarily from 7 to 8 weeks. The rats were then sacrificed, the body length determined, the contents of the gastro-intestinal tract removed, and the carcass prepared for analysis for calcium, either by ashing all of it, or by freezing and grinding to a homogenous sample, weighed portions of which were ashed.

The test foods and basal rations in each feeding experiment were also analyzed for calcium. The well-known McCrudden method for the determination of calcium in biological materials was used, with slight modifications, in this experiment.

EXPERIMENTAL RESULTS

Liquid vs. dried milk. The first experiment involving a biological comparison of the calcium in unpasteurized liquid skim milk and in "dry milk solids" gave results indicating a superiority of the liquid over the dried milk. The difference was slight (5.2%), but statistically significant, the probability

of its being a fortuitous result being only 0.02. It was thought that the indicated effect of desiccation might possibly be due to a destruction of vitamin C in the milk during its desiccation. It seemed conceivable that, though the rat could demonstrably synthesize the vitamin, it might not be able to do so at a rate sufficient to secure the full advantageous effects on calcium metabolism. Hence, a series of experiments to test this possibility was carried out, and, as the results were all negative in

TABLE 1

Experiments concerned with the effect of desiccation on the utilization of the calcium of milk

EXPERIMENT NUMBER	GROUPS OF RATS AVERAGED	CALCIUM SOURCES COMPARED:	AVERAGE FINAL CONTENT OF CALCIUM IN CARCASS	ESTIMATED AVERAGE CALCIUM RETENTIONS	PROBABILITIES
			%	mg.	
1	8	"Dry milk solids" + vitamin C	0.850	1152	0.082
		"Dry milk solids"	0.785	1046	
2	8	Liquid skim milk + vitamin C	0.599	496	0.29
		"Dry milk solids" + vitamin C	0.609	508	
3	6	"Dry milk solids" + vitamin C	0.722	628	0.14
		"Dry milk solids"	0.689	592	
4	12	"Dry milk solids" + vitamin C	0.980	542	0.28
		"Dry milk solids"	0.991	549	
5	12	Liquid skim milk	0.747	555	0.21 [1]
		Dried skim milk	0.745	565	0.38 [2]
		Dried skim milk + vitamin C	0.743	558	0.15 [3]

[1] Liquid skim milk vs. "dry milk solids."
[2] Liquid skim milk vs. "dry milk solids" + vitamin C.
[3] "Dry milk solids" vs. "dry milk solids" + vitamin C.

significance, other experiments were performed to attack again the original problem of the effect of desiccation on the biological utilization of milk calcium. As the summary of the most significant results of these experiments (table 1) clearly shows, no considerable evidence was secured that the value of milk calcium for the growing rat was to any appreciable extent impaired by commercial desiccation of the milk. Also, no advantage in calcium utilization was conferred upon any of

the experimental rations by the addition of ascorbic acid to it at the rate of 1 to 3 mg. per rat daily. The last experiment (no. 5) was particularly convincing, not only because of its scope, twelve trios of rats being used as subjects, but because particular pains were taken to insure against the destruction of the ascorbic acid before consumption by the rat. These experiments confirm the negative results of Henry and Kon ('39).

After the five experiments of this series were completed and their results studied and analyzed, the data of the first experiment, the only one giving positive indications, were re-studied and re-checked. The anomalous result appeared to depend upon one very questionable analysis of a sample of liquid milk. Hence, the data of this experiment have not been reported.

The evidence here presented to the effect that the desiccation of liquid milk does not appreciably impair its value as a source of dietary calcium, receives some support from recent experiments in human nutrition. Kinsman and associates ('39), in prolonged experiments on eleven pre-school children, report an average utilization of the calcium in "dry milk solids" of 19% and an average percentage retention of the same magnitude. For an adult subject, Steggerda and Mitchell ('39) obtained a percentage utilization of the calcium of "dry milk solids" of 20. To be compared with these values is the average retention of 19% of the dietary calcium, of which fresh milk contributed 80%, reported by Pierce et al. ('40) for a group of ten pre-school children. These results indicate the existence of no appreciable difference in the availability of the calcium in fresh and in dried milk. The experiments of Kramer, Latzke and Shaw ('28) on the calcium and phosphorus metabolism of five children and seven adults receiving diets in which most of the calcium was provided by fresh, pasteurized, evaporated or dried milk, yielded quite contrary results, in that dried milk quite consistently proved a definitely poorer source of available calcium than fresh milk. Since the experiments involved a succession of short (6 day) metabolism periods, in which the different kinds of milk were tested, with

no intermediate adjustment periods, there is no assurance that the balances obtained are representative of the diets consumed.

"Dry milk solids" vs. carrots, fresh and steamed. The more significant average results of the three experiments involving a comparison of the calcium of "dry milk solids" with the calcium of fresh and steam-cooked carrots are summarized in table 2.

In experiment 1, the basal diet contained dried extracted whole egg as a source of protein and was hence fairly high in calcium content. The test foods supplied only 56% of the total intake of calcium. The amounts of calcium retained, both in milligrams and in percent of the intake, were greater in all fourteen pairs for the rats receiving the milk powder supplement. There can thus be no question of the significance of the difference as indicating a better utilization of milk calcium than of the calcium in fresh carrots. The average percentage retentions were 83.0 for the milk diet and 71.2 for the carrot diet. Thus the fresh carrot diet calcium was only 86% as well utilized as the milk diet calcium.

Experiment 2 consisted of a trio comparison of the calcium in milk solids and in raw and cooked carrots, eight trios of rats being used. In this experiment, because of the use of a basal diet containing a lower concentration of calcium, the test foods supplied 86% of the total calcium consumed. The average retention of calcium was considerably greater for the milk diet than for the carrot diets, being 467, 394, and 397 mg. for the milk, fresh carrot, and cooked carrot supplements, respectively, or 91.0, 76.7, and 77.3% of the consumed calcium. A statistical analysis by the method of Student ('08) reveals significant average differences between the milk diet and the fresh carrot diet ($M = 69.5$ mg., $s = 37.8$ mg., $P = 0.0046$) and between the milk diet and the cooked carrot diet ($M = 68.2$ mg., $s = 38.1$ mg., and $P = 0.0051$), but obviously an insignificant average difference between the fresh carrot diet and the cooked carrot diet. The values for P are the probabilities on a scale of one that the fortuitous factors alone would produce an average difference as large as, or larger than, that actually

TABLE 2

Average results of the experiments comparing the calcium of milk with that of carrots, lettuce and green string beans

	EXPERIMENT 1		EXPERIMENT 2			EXPERIMENT 3		EXPERIMENT 4		EXPERIMENT 5		
	Milk solids	Fresh carrots	Milk solids	Fresh carrots	Cooked carrots	Fresh carrots	Cooked carrots	Milk solids	Fresh lettuce	Milk solids	Fresh string beans	Canned string beans
Number of pairs or trios	14	14	8	8	8	6	6	10	10	12	12	12
Average daily gain, gm.	1.92	1.86	1.86	1.85	1.90	1.73	1.70	1.65	1.62	1.74	1.71	1.72
Attained body length, mm.	184.1	184.6	188.3	187.0	188.0	186.7	186.2	184	182	182	180	181
Calcium intake, mg.	713	713	522	522	522	481	481	500	500	419	419	423
Calcium intake in test food, %	56	56	86	86	86	85	85	80	80	90	90	90
Ash content of carcass, gm.	3.68	3.44	3.49	3.34	3.30	3.21	3.18	2.94	2.73	2.92	2.68	2.63
Ash content of carcass, %	2.78	2.64	2.37	2.32	2.25	2.34	2.30	2.33	2.20	2.57	2.41	2.34
Calcium content of carcass, mg.	952	872	836	754	762	764	744	665	584	687	588	579
Calcium content of carcass, %	0.719	0.671	0.585	0.543	0.536	0.552	0.543	0.526	0.470	0.605	0.528	0.516
Retained calcium, mg.	591	508	467	394	397	379	359	385	308	349	257	245
Percentage retention of calcium	83.0	71.2	91.0	76.7	77.3	78.5	74.3	76.8	61.4	83	61	60
Phosphorus content of carcass, mg.	—	—	—	—	—	—	—	—	—	569	528	512
Phosphorus content of carcass, %	—	—	—	—	—	—	—	—	—	0.501	0.474	0.456
Retained phosphorus, mg.	—	—	—	—	—	—	—	—	—	313	277	259

observed. The evidence seems clear in this experiment also that the calcium of milk is definitely better utilized by the growing rat than the calcium of fresh carrots, and, furthermore, that cooking carrots by steaming does not alter appreciably their value as a calcium food. If the calcium in the milk diet is given a value of 100, the values of the calcium of fresh carrots and cooked carrots are, respectively, 84 and 85.

In experiment 3, another comparison was made between fresh and cooked carrots, using six pairs of rats and rations such that 85% of the total calcium content was provided by the test foods. The mean difference (M) in stored calcium for the two diets was 20.0 mg., the standard deviation (s) was 24.8 mg., and the probability of a chance outcome is 0.065, too large to be neglected according to current biometrical practice. While the experiment thus offers some evidence for a better utilization of the calcium of fresh carrots than of the calcium of cooked carrots, it falls considerably short of a demonstration to that effect.

The fresh carrots used in the above experiments contained on the average 0.0325% of calcium and 0.0398% of phosphorus, and the dry skim milk, 1.200% calcium and 1.022% phosphorus.

"Dry milk solids" vs. lettuce. The relative value of the calcium in "dry milk solids" and in lettuce (containing 0.0140% calcium) was tested in experiment 4, using ten pairs of growing rats. Eighty percent of the calcium intake was contained in the test foods. The gains in body weight were slow, averaging only 1.63 gm. daily, and the percentage retentions were correspondingly, and perhaps consequently, low, averaging 76.8 for the milk diet and 61.4 for the lettuce diet. The mean difference in calcium retention was 77.5 mg., the standard deviation of differences was 31.2, and the probability of a fortuitous difference (Student, '25) was 0.00002, so very small that it may be neglected. As further evidence of the superiority of the milk supplement, the average body length of the milk rats was significantly ($P = 0.025$), if only slightly ($M = 1.4$ mm.), greater than that of the lettuce rats.

In this experiment the lettuce calcium was only 80% as well utilized as the milk calcium.

"Dry milk solids" vs. green string beans. The relative values in nutrition of the calcium in "dry milk solids" and in fresh and commercially canned green beans were determined in experiment 5, using twelve trios of rats. In this experiment, the phosphorus metabolism as well as the calcium metabolism was measured. As with the carrot experiment, the difference in calcium utilization was statistically significant between the milk solids and the raw vegetable ($P = 0.00001$), but was quite insignificant between the raw and the canned vegetable. The retention of phosphorus was also distinctly better on the milk diet than on the fresh string bean diet ($P = 0.0062$). In this experiment the test foods provided 90% of the total calcium consumed.

That the milk diet promoted a more rapid calcification of the bones than the vegetable diets, is also shown by the higher ratio of retained calcium to retained phosphorus, these ratios being 1.11 to 1 for the milk diet, 0.93 to 1 for the fresh bean diet and 0.95 to 1 for the canned bean diet. The calcium in the bean diets was only 73% as available to the growing rats as the calcium in milk solids.

DISCUSSION

The calcium of the vegetables tested in this study is quite definitely less available to the growing rat than the calcium of milk. Since considerable differences have not been found in the utilization by animals of calcium salts appreciably soluble in water (Bethke, Kennard and Kick, '29; Deobald, Elvehjem, Hart and Halpin, '36; Mitchell, Carroll, Hamilton, Garrigus and Hunt, '37) if adequate amounts of vitamin D are provided, and since a number of calcium salts, both organic and inorganic, have been shown to be as valuable in nutrition as the calcium compounds of milk (Stearns and Jeans, '34; Steggerda and Mitchell, '39; Pierce et al., '40), it would appear that the differences noted between milk and vegetables are the result of the presence in vegetables of constituents, possibly

carbohydrate in nature, that impair calcium absorption from the intestinal tract.

These constituents may act by rendering the calcium compounds in vegetables less accessible to the solvent action of the digestive juices, in much the same fashion as the indigestible carbohydrates impair the digestion of cereal and legume proteins (Mendel and Fine, '11a; '11b; '11c; '12). However, the fact that the cooking and commercial canning processes, that would be expected to rupture cellular membranes, do not improve the utilization of vegetable calcium, would militate against this explanation.

A second possibility is that the constituents of vegetables responsible for the impairment in calcium utilization act either by combining with the calcium to form insoluble compounds, as the oxalates of foods are known to do, or to modify unfavorably the reaction of the intestinal contents, or to increase intestinal motility. These factors must account for the so-called "associative effects" of foods in digestion, whereby one food will impair the digestibility of another. If this possibility is correct, then not only will the calcium of vegetables be less completely utilized than the calcium of milk, but the presence of vegetables in the diet will depress the absorption of the calcium compounds in foods with which they are fed, even in milk.

Evidence favoring the latter explanation may be found in the results of experiments 1 and 2 (table 2). In experiment 1, fresh carrots furnished only 56% of the calcium intake, the remainder being provided to a considerable extent by dried egg. In experiment 2, fresh carrots provided 86% of the calcium intake. Nevertheless, the percentage retention of calcium was approximately the same, 71.2 and 76.7, respectively, and in comparison with the calcium of the milk diets, the calcium of the carrot diets was of equal value, i.e., 86 and 84% as good, respectively. Apparently, the addition of the non-calcium constituents of carrots to the basal diet in experiment 1, impaired the utilization of the calcium of the carrots and of egg to approximately the same extent. Tso ('26) has re-

ported experimental observations indicating a very high utilization of the calcium of egg.

SUMMARY AND CONCLUSIONS

In a series of ten experiments on growing rats, involving equalization of calcium intakes and of gains in body weight among comparable animals, the calcium retention was determined by carcass analysis following a period of experimental feeding sufficiently long to permit gains in weight of 100 gm. The availability of the calcium of various vegetable foods and of unpasteurized liquid milk was compared in each case with that of the calcium of "dry milk solids" at levels of calcium intake insufficient to promote maximum calcification of the bones. The results obtained justify the following conclusions:

1. The commercial desiccation of milk does not appreciably impair the value of its calcium in the nutrition of growing animals.

2. The calcium of milk is definitely better utilized than the calcium of the vegetables tested. Under the conditions of these experiments, the calcium of fresh carrots, fresh lettuce and fresh green string beans was 85, 80, and 74%, respectively, as available as the calcium of milk.

3. The steam cooking of carrots and the commercial canning of green string beans do not modify appreciably the value of these vegetables as sources of dietary calcium.

4. The constituents of vegetables tend to depress the utilization of the calcium of other foods with which they are fed. The maximum extent of this effect may be determined by the extent to which the calcium of the vegetable, when fed as the sole source of calcium, is utilized in comparison with the calcium of milk.

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THE UTILIZATION OF THE CALCIUM OF DI-CALCIUM PHOSPHATE BY CHILDREN [1,2]

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ONE FIGURE

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INTRODUCTION

Recent studies carried out in these laboratories have yielded data which show clearly that man is remarkably uneconomical in his utilization of milk calcium. In the carefully controlled experiments of Kinsman, Sheldon, Jensen, Bernds, Outhouse and Mitchell ('39), the well-nourished subjects were able to use only one-fifth of the calcium eaten in this form. Such wastage with respect to a food which has long been considered superior as a conveyer of calcium would warrant further investigation of other sources which may, possibly, yield their calcium more readily. The question arises as to whether or not the pure calcium salts, which are frequently recommended by physicians as a supplement to the diet, are better than milk in this respect. Inasmuch as di-calcium phosphate gave good calcium retention in the babies studied by Stearns and Jeans ('34–'35), this salt was selected for the present study. If its calcium were unusually well utilized, this inorganic compound

[1] A preliminary report of some of these data was given before the American Public Health Association, Kansas City, October, 1938, and before the American Institute of Nutrition, New Orleans, March, 1940.

[2] Aided by a grant from the American Dry Milk Institute, Inc., Chicago.

[3] A portion of these data was presented by Miss Herta S. Breiter in partial fulfillment of the requirements for the degree of Master of Science at the University of Illinois, June, 1939.

would be a valuable adjunct to human dietaries in view of its relatively high calcium and phosphorus content, its palatability and its low cost.

EXPERIMENTAL

The subjects used in this experiment were six pre-school boys who were well adapted to the demands of the experiment since they had participated previously in a study of the utilization of milk calcium. For 9 weeks immediately preceding the present study, they had been fed calcium at a generous level (i.e., 900 mg. daily) in an attempt to saturate their body stores with this element. Throughout all these metabolism studies, totalling 54 weeks, the subjects were maintained on a constant regimen. This regimen was essentially the same as that described in a previous study with girls (Outhouse, Kinsman, Sheldon, Twomey, Smith and Mitchell, '39); however, an increase in the quantities of cereals and potatoes was necessary in order to satisfy the higher caloric needs of these boys who were heavier than the girls. The ages, heights and weights of the boys at the end of period VI and their gains in weight during the 12 months preceding the end of the study are given in table 1. The procedures for the collection of metabolic materials were identical with those of the previous studies; this was also true for the analytical techniques.

Inasmuch as the purpose of this study was the securing of a quantitative measure of the availability for growth purposes of the calcium of a specific compound, a procedure was used which would give a value that would be uninfluenced by the subject's maintenance requirement for calcium. This procedure, proposed by Mitchell in a previous study on milk calcium (Kinsman, Sheldon, Jensen, Bernds, Outhouse and Mitchell, '39), consists of determining calcium balances at two levels of intake, the difference in the levels being due to the calcium content of the substance under investigation. In computing percentage utilization, the difference between the calcium intakes at these two levels and the difference between calcium retentions were used. By considering only the incre-

ment in retention which was induced by the increment in intake, one can differentiate between the retention relating to the total intake and the retention resulting from the ingestion of the test substance. In this laboratory, the latter is interpreted as being representative of utilization.[4]

Obviously, this procedure necessitates that both levels of calcium intake be sufficiently generous to allow for positive balances (i.e., they must exceed the subject's maintenance requirement) but not for maximal storage of calcium. Since, in previous studies on these same, and other, children, a daily intake of 500 mg. did not result in maximal calcium retentions, this amount of calcium was chosen as the higher level in the present experiment; of this total intake, 126 mg. were fed in the form of $CaHPO_4$. The calcium salt was weighed in weekly allotments of exactly 3.8 gm. for each child; this amount was divided into seven approximately equal parts each of which was placed in a gelatin capsule. One capsule was given each morning at breakfast. This procedure was continued until balances for an additional week did not change appreciably the values which were obtained on averaging the calcium balances for consecutive preceding weeks; this time interval (i.e., 7 weeks) is designated as period VI. Subsequent to this period, calcium was fed at a lower level; this low level of intake was achieved by removing the supplement of $CaHPO_4$. The length of this period (period VII) varied for the different subjects; it ranged from 7 to 13 weeks. During the first few weeks, subjects Br and Jw retained such small quantities of calcium that it was deemed advisable to discard the data for those weeks and to continue the experiment for at least 6 weeks after the retentions had become more satisfactory and more constant. Subjects R and G withdrew from the experiment at the beginning of this period; therefore, it was neces-

[4] In this discussion use is made of the terms "per cent retention" and "per cent utilization"; they are not synonymous since per cent retention values will be lower than per cent utilization values due to endogenous losses. However, for children who apparently have little or no maintenance requirement, these terms are probably interchangeable.

sary, in computing percentage utilization, to use the values observed for these two boys during an earlier period (period II). Because the respective sources and intake levels of calcium for periods II and VII were identical, such a substitution seemed reasonable.

DAILY RETENTIONS OF CALCIUM

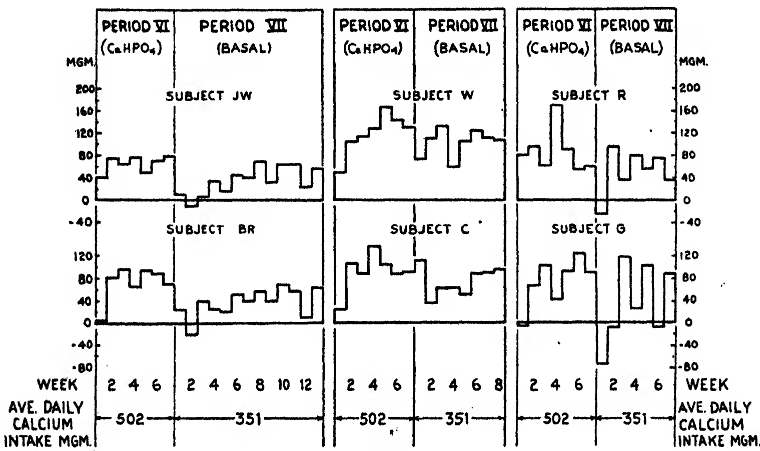

FIGURE I

RESULTS

The data pertaining to this study are recorded in figure 1 and in table 1. During the period in which the subjects received the basal diet, the daily calcium intakes ranged from 328 to 364 mg. with an average of 351 mg. For the individual subjects R, W, C, G, Br and Jw, these intakes were 328, 359, 364, 343, 357 and 353 mg., respectively. The corresponding average daily retentions were 63, 107, 69, 53, 48 and 45 mg., and averaged 64. When these retentions are expressed as a percentage of the intake, they average 18.3 with individual values of 19.2, 29.8, 19.0, 15.5, 13.5 and 12.8, respectively, for the subjects in the order listed above.

When di-calcium phosphate was fed, the calcium intakes averaged 502 mg. daily. Subjects R, W, C, G, Br and Jw re-

tained, as daily averages, 89, 131, 103, 87, 83 and 69 mg., respectively, on intakes of 483, 493, 512, 517, 512 and 492 mg. These retentions represent, respectively, 18.4, 26.6, 20.1, 16.8, 16.2 and 14.0% of the intakes; the average value for the group is 18.7%.

As stated in a preceding paragraph, the method adopted in this study for computing the percentage utilization of the calcium in $CaHPO_4$ makes use of the difference in the quantity

TABLE 1

Data on the utilization of the calcium of di-calcium phosphate

SUBJECTS		R	W	C	G	Br	Jw	Ave.
Age in years at end of period VI		3.5	4.5	5.6	6.1	6.5	6.5	
Weight in kg. at end of period VI		17.4	18.4	21.3	21.2	23.2	21.1	
Height in cm. at end of period VI		102	109	117	117	122	124	
Weight gain (ave. daily) in gm. during 12 mo. prior to end of period VII		7.2	10.2	6.4	5.4	4.4	5.8	
$CaHPO_4$	Days on experiment [1]	42	42	42	42	42	42	
+ basal	Calcium intake (ave. daily) in mg.	483	493	512	517	512	492	502
diet	Calcium retention (ave. daily) in mg.	89	131	103	87	83	69	94
(period VI)	Calcium retention, %	18.4	26.6	20.1	16.8	16.2	14.0	18.7
Basal	Days on experiment [1]	42	49	49	42	56	70	
diet	Calcium intake (ave. daily) in mg.	328	359	364	343	357	353	351
(period VII)	Calcium retention (ave. daily) in mg.	63	107	69	53	48	45	64
	Calcium retention, %	19.2	29.8	19.0	15.5	13.5	12.8	18.3
Utilization of calcium of $CaHPO_4$		16.8	17.9	23.0	19.5	22.6	17.3	19.5

[1] Exclusive of first week except for subjects Br and Jw who required 5 and 3 weeks, respectively, for adjustment to the low level of intake of period VII.

of calcium retained during the basal period and during the $CaHPO_4$ period and of the difference in the quantity of calcium ingested during the two periods. In the data presented in the table, it will be noted that the difference in the average calcium intakes of periods VI and VII was 151 mg. rather than 126 mg.—the amount of calcium furnished by the $CaHPO_4$. In other words, less food calcium, to the extent of 25 mg., as an average, was eaten during the basal period.

This unforeseen deviation from the experimental plan to have the difference in calcium intakes due solely to the $CaHPO_4$ supplement can be accounted for almost entirely by an increase in the consumption of bread by every child during the time he was ingesting $CaHPO_4$. Although the bread was made with water and contained only 0.033% calcium, it contributed a significant and variable amount of calcium to the diet since the children were allowed to eat it ad libitum as a means of satisfying individual caloric needs. Consequently, the difference in retention of calcium between periods VI and VII was not due entirely to the ingestion of $CaHPO_4$; approximately one-sixth of the difference in calcium intake must be attributed to the constituents of bread. On the basis of this mixture of calcium, 83% of which was derived from $CaHPO_4$, the following utilization values were obtained for subjects R, W, C, G, Br and Jw: 16.8, 17.9, 23.0, 19.5, 22.6 and 17.3%, respectively. These values average 19.5%.

DISCUSSION

The six pre-school boys who served as subjects in this study made poor use of the calcium of di-calcium phosphate; i.e., they retained only approximately 20% of the calcium of this salt when it was fed under conditions which were designed to demonstrate maximal calcium utilization. Such great wastage of the ingested calcium does not necessarily contraindicate the use of this inorganic salt as a source of calcium for children, inasmuch as equally great calcium wastage was observed in these same subjects during an earlier study in which milk supplied an almost comparable quantity of calcium (Kinsman et al., '39). The percentage utilization of milk calcium by subjects R, W, C, G, Br and Jw proved to be 17.8, 21.5, 20.0, 12.7, 19.1 and 27.5, respectively. Although each subject did not make use of exactly the same amount of calcium from both sources, the utilization values are of the same order of magnitude, and the averages for the two groups are almost identical —i.e., 19.5% and 19.8%. This finding—that there is no essential difference in the extent to which the calcium in these two

substances is utilized by children—agrees with the conclusions drawn by Stearns and Jeans ('34–'35) from their study of pre-school children. Jansen ('24) reached a similar conclusion in his study on an adult. These are the only experiments which have been found in the literature in which milk and di-calcium phosphate were compared.

However, other calcium salts have been fed to children of the same age-range as those of the present study. Stearns and Jeans ('34–'35) fed the calcium salts of di- and tri-phosphates, lactate, carbonate and gluconate, singly or in conjunction with milk, but neither percentage retention nor percentage utilization values can be computed from their data since they were reported as group averages. These investigators felt that there was no difference in the availability of the calcium of these salts with the exception of gluconate calcium; two 6-year-old children were in negative balance when they received calcium gluconate. (However, the children on the 7 to 12 age-group apparently were able to make just as good use of this salt as they did of milk or of $CaHPO_4$.) One of Grosser's ('20) subjects was a pre-school child—he was 2¾ years old; his utilization of the calcium of calcium lactate amounted to 24%. But, in view of the short duration of both of these experiments, little weight can be placed on the data.

That growing children, under certain conditions, might not exhibit an appreciable maintenance requirement for calcium was suggested in a previous publication (Kinsman et al., '39). This belief is based on two facts; namely, that in the eleven children studied (a) no consistent difference existed between the values for percentage retention and those for percentage utilization and (b) the values for percentage retention at different levels of intake, instead of increasing with increasing calcium intake, remained constant. The data of the present study carry the same implication. Although the average values for percentage retention during periods VI and VII are lower than the average value for percentage utilization of the calcium in $CaHPO_4$, this difference is not found consistently for the individual subjects. This inconsistency is obvious in the

following comparison of values for percentage retention and percentage utilization of 18.4 and 16.8, 26.6 and 17.9, 20.1 and 23.0, 16.8 and 19.5, 16.2 and 22.6 and of 14.0 and 17.3, respectively, during period VI for subjects R, W, C, G, Br and Jw. Moreover, the average values for percentage retention of the calcium ingested during period VI and VII are practically identical; i.e., they are 18.3 and 18.7 for the respective periods. Such an analysis of the data herein presented supports the contention that, at levels of intake which do not exceed the minimal requirement for calcium for maximal storage, growing children show little, if any, requirement for calcium for maintenance purposes. The practical inference arising from this belief is the previously expressed suggestion that, in formulating the child's dietary, insofar as calcium is concerned, only the requirement for growth purposes probably need be considered.

SUMMARY AND CONCLUSIONS

Six little boys, ranging in age from 3½ to 6½ years, were fed two levels of calcium, both of which were below the minimal requirement for maximal retention but obviously in excess of a possible maintenance requirement. The lower level contributed, as an average, 350 mg. of calcium daily, the higher one, 500 mg. daily; the difference was due, mainly, to the daily supplementation of the basal dietary with 543 mg. of di-calcium phosphate. The utilization of the calcium averaged 19.5%; individual values were 16.8, 17.9, 23.0, 19.5, 22.6 and 17.3%. These same children had previously served as subjects in a study of the availability of milk calcium and had been able to utilize only 19.8% of that calcium; therefore, it may be concluded that di-calcium phosphate is not superior to milk as a source of calcium for children and that, except for the person who is allergic to milk, there is little virtue in recommending the replacement of milk in the diet by di-calcium phosphate.

ACKNOWLEDGMENT

Grateful appreciation is herewith expressed for the cooperation extended by Mrs. Charlotte Fitzgerald, superintendent, and the members of the Board of Trustees of the Cunningham Children's Home, where this experiment was conducted, and by Miss Charlotte Beard and Miss Nellie Ratcliffe, dietitians. The authors also wish to acknowledge their indebtedness to Dr. H. H. Mitchell, of the Animal Husbandry Department, University of Illinois, for his continued interest in and support of these studies; his counsel has been invaluable.

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PECTIC ENZYMES

V. THE FATE OF PECTINS IN THE ANIMAL BODY [1]

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ONE FIGURE

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For several reasons the fate of pectins in the animal body is of interest. The pectins of various fruits and vegetables are a part of the regular diet and although they are present in small quantities it would be of importance to know whether they are utilized as certain other polysaccharides are. Pectin is also an important component of the apple diet often successfully employed to combat diarrhea. The usefulness of this treatment is generally recognized (Council on Foods, American Medical Association, '37) but its working mechanism has not yet been satisfactorily explained. No final answer to this question can be expected until the metabolic fate of the various components of apples is clear.

All investigators who have studied this question agree that pectin added to the diet of animals and human subjects cannot be recovered from the feces. Fürth and Engel ('31), Manville, Bradway and McMinis ('36) and others believe that pectin is a digestible carbohydrate, hydrolysed and utilized in the animal body, but there is no evidence supporting this assumption. Murer and Crandall ('40) suggest that pectin is not hydrolysed in the digestive tract of the dog.

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An investigation of the working mechanism of the apple diet was begun at this Experiment Station some time ago and in connection with it various tests were conducted to obtain information on the fate of pectins in the animal body. The results obtained are presented herewith. A method for the determination of possible slight decomposition of pectins is also described with the hope that it will facilitate further investigations in this field.

METHODS AND MATERIALS

The powdered citrus pectin used was exhaustively extracted with cold 50% alcohol and contained 88.4% uronic acid (Lefèvre and Tollens, '07, expressed as polygalacturonic acid) and 10.28% CH_3O determined by saponification. It also contained 0.49% ash, and 1 gm. of the pectin gave 1.09 gm. of calcium pectate (Carré and Haynes, '22). The pectin was thus composed almost exclusively of polygalacturonic acid in which most of the carboxyl groups were esterified.

It may be noted that a preliminary precipitation of the pectins with acidified alcohol is usually desirable. The alcohol precipitate is dissolved in a small volume of hot water and this solution used for the determination of calcium pectate and also of the relative viscosity by an Ostwald viscosimeter at 30°C.

In these experiments it was necessary to use a method by which any possible small digestion could be determined on a portion of the pectin applied. It has been reported previously (Kertesz, '39) that upon the slightest decomposition of pectin, there is a great decrease in its viscosity. This causes the ratio $\frac{\text{calcium pectate}}{\text{relative viscosity}}$ to increase materially. The method is applicable only in cases of slight decomposition because upon extensive hydrolysis the calcium pectate precipitate disappears completely.

In all in vitro experiments 30°C. was used rather than body temperature because the degeneration of the viscous properties of pectin is slower at this temperature. There was also less likelihood of slow heat inactivation of the various en-

zymes at this temperature during prolonged digestion. Finally it was also desirable to have the reactions proceed at the same temperature at which the viscosity determinations were performed. In order to avoid hydrolysis or saponification of the pectin by the medium, all in vitro experiments were made at pH 3.4 and 6.4.

EXPERIMENTAL RESULTS

The observation that pectin added to the diet is digested in the body was substantiated by several experiments on dogs as well as on human subjects. In not a single case could any pectin be found in the feces, indicating that it was completely hydrolysed. To discover the agencies responsible for this hydrolysis various digestive secretions were examined for their effect on pectin.

Saliva collected from human subjects, dogs and cows had no effect on pectin at pH 3.4 and 6.4 during several weeks of incubation in the presence of toluol.

Jejunal juice was collected from an isolated auto-transplanted segment in a dog; about 12 ml. were obtained in 4 hours. It contained very active saccharase, amylase and peptidase. To test its possible pectolytic activity 0.5 ml. portions of it were digested with 5.0 ml. of a 1.5% pectin solution in the presence of toluol for several days. Tests performed at various times up to 3 days gave no indication of the slightest decomposition.

Attempts to digest pectin in the stomach and small intestine of the dog failed. The dog used in this experiment weighed 14 kg. and had an enterostomy in the lower end of the jejunum. Previous experiments on the same dog indicated that, of 48 gm. available carbohydrate in test meal ingested, only 15 to 20% could be recovered from the fistula. With the aid of a stomach tube 45 ml. of a 2.0% solution of pectin, followed by about 25 ml. of water, was introduced. The dog showed no signs of discomfort.

At intervals of 60, 90 and 160 minutes 36 ml., 41 ml. and 10 ml. liquid respectively were obtained. In the combined

samples the pectin was precipitated and washed with alcohol, dried at room temperature and dissolved in water; 98.8% of the pectin introduced was recovered. The relative viscosity of this solution when compared with that of the original solution of the same pectin content gave no indication of hydrolysis.

In order to determine whether pectin is digested in the human stomach 200 ml. of a 1.23% solution of pectin was ingested by an adult male. Several samples of 13 to 25 ml. were

TABLE 1

Digestion of pectin in the human stomach and by pectinase

NO.	SAMPLE	DURATION OF EXPERIMENT	PECTIN PURIFIED FROM THE STOMACH CONTENTS OR DIGEST: Calcium pectate	Relative viscosity (water = 1)	Calcium pectate Relative viscosity (1)	CALCIUM PECTATE RELATIVE VISCOSITY OF REFERENCE SOLUTION OF SAME PECTIN CONTENT (2)	DIFFERENCE BETWEEN (1) AND (2)
		minutes	%				
1	Recovered by stomach tube	45	0.230	2.11	0.109	0.102	+0.007
2	Same as no. 1	90	0.137	1.79	0.077	0.081	−0.004
3	Incubated with juice from stomach	1440	0.393	3.25	0.121	0.117	+0.004
4	Vomited	30	0.117	1.46	0.076	0.071	+0.005
5	Digested with 0.1% commercial pectinase	5	0.452	2.52	0.179	0.129	+0.050
6	Same as no. 5	15	0.428	2.01	0.213	0.129	+0.084

recovered by stomach tube at intervals up to 90 minutes. The person on whom the experiments were performed was accustomed to swallowing the stomach tube and not the slightest discomfort was indicated by him from this operation or from any effect of the pectin. The samples obtained were examined separately and the results are shown in table 1. This table also shows the results obtained in an experiment in which a 5 ml. sample of juice taken from the stomach of the same person was incubated with 10 ml. pectin solution for 24 hours.

All results indicate the absence of digestion. Experiments with pectin ingested by an adult and vomited after 30 minutes also gave negative results.

Tests were conducted to determine the possible digestion of pectin by trypsin, pepsin, rennet and pancreatic amylase. Analyses of the reaction mixtures after 12 days' incubation showed that the pectin was intact.

To study the effect of feces on pectin a suspension was made of human feces and the mixture was filtered through cloth to remove the larger particles. From this suspension (containing 6.31% solids) 50 ml. aliquots were mixed with 25 ml. of a 1.25% pectin solution and the mixture incubated at 30°C. At

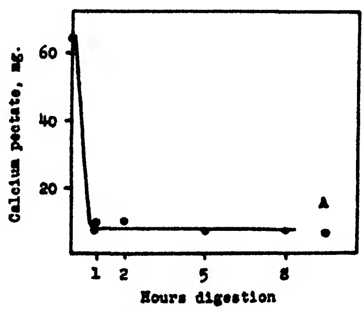

Fig. 1 Digestion of pectin with feces suspension. Sample "A" was digested with feces suspension plus 1% very active pectinase for 48 hours.

intervals up to 8 hours the digests were mixed with 300 ml. of 95% alcohol, the precipitate was dissolved in hot water, made up to volume and an aliquot representing one-fifth of the original mixture was used for the precipitation of calcium pectate. The results obtained are presented in figure 1. The pectin was rapidly digested by the feces suspension. Under these conditions a small quantity of material present in the feces suspension gave a precipitate which did not disappear after prolonged digestion with pectinase. The pectin was also decomposed by feces in the presence of toluol although the progress of the digestion was irregular and much slower in

this case. Canine feces gave results similar to those shown in figure 1.

DISCUSSION

Saliva and the secretions of human and canine stomachs as well as the small intestine of dogs, are devoid of enzymes acting upon pectin. On the other hand pectin incubated with a suspension of feces is rapidly decomposed. This suggests that the pectin passes through the esophagus, stomach and small intestine without decomposition but is subsequently decomposed by the enzymes produced by the abounding flora of microorganisms rather than by enzymes excreted by the large intestine.

Obviously the validity of these conclusions depends on the acceptance of certain assumptions. There is some doubt as to whether the observations made on dogs may be used in predicting the fate of pectin in human beings. Nutritional and other conditions may also be of importance. Murer and Crandall ('40) mention that pectin fed to fasting animals is mostly excreted. Excretion of pectic materials is also apparent when the apple diet is used on patients (or animals) suffering from diarrhea. Perhaps in such cases the normal bacterial flora in the large intestine is altered; this results in a failure to decompose the pectin. There is little excretion of enzymes in the large intestine and it appears safe to assume that the pectin reaching the colon is decomposed there by bacterial enzymes.

The matter of carbohydrate absorption from the large intestine is still a controversial subject. The observations of Imhäuser ('32) that pectin orally administered to dogs does not increase the blood sugar make it probable that the galacturonic acid derived from the pectin is fermented rather than absorbed. Imhäuser also states that the decomposition products of pectin were not excreted. Tests conducted on the fermentation of pectin and galacturonic acid by various intestinal microorganisms (Saunders and Kertesz, '40) indicate that most microorganisms capable of hydrolysing pectin also ferment galacturonic acid.

It is not within the scope of this article to discuss the significance of the present findings in regard to the working mechanism of the apple diet. But it may be suggested that if pectin is not decomposed until it reaches the large intestine its therapeutic action may depend on its colloidal properties. The galacturonic acid derived from the pectin upon hydrolysis may be available for detoxication (Manville, Bradway and McMinis, '36) after absorption from the colon. It is also possible, although not very probable, that some galacturonic acid would reach the ileum by regurgitation (Manville, '40), be absorbed and participate in detoxication reactions.

The author wishes to express his appreciation to Dr. E. S. Nassett of the University of Rochester and to Dr. J. A. Dye of Cornell University for their kind cooperation in performing some of the experiments reported in this paper.

SUMMARY

Tests made with human subjects and dogs indicated that saliva and gastric juice do not contain enzymes acting upon pectin. Pectin passed through the stomach and part of the small intestine of a dog could be recovered without loss. Trypsin, pepsin and rennet had no effect on pectin in vitro, but pectin incubated with feces was rapidly decomposed.

It appears probable that pectin taken orally is not attacked until it reaches the large intestine where it is completely hydrolysed by bacterial enzymes.

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RELATIONSHIP BETWEEN INSENSIBLE LOSS OF WEIGHT AND HEAT PRODUCTION OF THE RABBIT

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ONE FIGURE

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Benedict and Root ('26) and Newburgh et al. ('37) have shown that with humans there is a relationship between the insensible loss of weight and the heat production. Benedict and Root state that measurement of the insensible loss of weight alone can be used to estimate the heat production. Gasnier and Mayer ('34) studied the insensible loss of weight of rabbits at 20°C. and found that this loss decreased with fasting, was different with different animals, increased as the environmental temperature rose, and was influenced by other conditions. It is the purpose of this paper to determine to what extent the rabbit's insensible loss of weight at a given temperature is related to its heat production, to note factors that may affect this relationship, and to determine whether measurement of the insensible loss can be used to estimate this animal's heat production.

METHODS

Twelve adult rabbits of various sizes were used in these experiments.[1] The metabolism measurements were made at 28° to 29°C., with the apparatus used by Lee ('39), and for 24 hours beforehand the rabbits were kept at 28°C. A rabbit was weighed in a wire basket on a 5-kg. Sauter balance. It was

[1] The author gratefully acknowledges the technical assistance of Mr. George Lee.

then transferred to the metabolism chamber, and when the chamber had been sealed and ventilated for 3 minutes, the measurements of the gaseous exchange began. There were nine consecutive periods of such measurements, each of 20 minutes' duration. The rabbit was then removed from the chamber and weighed again in the wire basket. The measurement of the insensible loss of weight covered about 3⅓ hours and that of the metabolism 3 hours. The heat production was calculated from the measurements of the respiratory exchange.

At the beginning of the first period of the respiration experiment equilibrium of the gaseous mixture inside the chamber was probably not fully established, but the metabolism measurements were started early in an effort to have the time covered by such correspond as nearly as possible to the time of measurement of insensible loss. The transfer of the rabbit from the weighing basket to the chamber undoubtedly caused an increased metabolism, and it was desired to include this period in the metabolism measurement, as it was included in the measurement of the insensible loss. The heat production in the first period of each metabolism experiment averaged 13% above the average heat production in all the periods of the experiment.

Only measurements obtained when the rabbit was dry at the start and end of the experiment, when no urine or feces were voided, and when there were no visible indications of moisture from condensation, drooling, or any other source, have been included in the results reported in this paper.

As the ventilating air supplied to the chamber was dried by calcium chloride, the relative humidity of the outcoming air at the average chamber temperature was calculated from the hourly insensible loss (corrected for the carbon-dioxide elimination and the oxygen absorption to obtain the grams of water vaporized per hour) and the volume of air ventilating the chamber per hour. The air entered the chamber near the rabbit's head. Hence because of the slow ventilation rate and because calcium chloride cannot completely dry air, the

average humidity of the chamber was nearly that calculated for the outcoming air.

RESULTS

In table 1 are recorded the results of seventy-four experiments in which simultaneous measurements were made of the heat production and the insensible loss of weight of these twelve rabbits. In six instances the rabbits were measured immediately after eating but in all other instances had been 24 hours without food at the time of measurement and were in the post-absorptive state, as indicated by their respiratory quotients (range, 0.71 to 0.78). When the values for insensible loss of weight per hour are plotted with reference to the hourly heat production under post-absorptive conditions (fig. 1), it is apparent that there is a straight-line relationship between these two factors.

Humidity. As the insensible loss is made up in large part of evaporated water and as evaporation of water is affected by differences in humidity, the question arises as to whether this factor influences the relationship under consideration. With eight rabbits the humidity differed by 20% or more in different fasting experiments, but comparison of the results obtained at the extremes of humidity indicates no tendency for the relationship to be influenced by humidity. When the values for humidity were indicated against the plotted points, the distribution of the high and low humidities above and below the average curve showing the general trend of the data was essentially even. In three experiments (indicated by crosses in fig. 1) in which calcium chloride was placed in the bottom of the respiration chamber and the rabbits rested on a wire grate slightly above it, the humidity of the air, at least in parts of the chamber, was extremely low. These showed an appreciably greater insensible loss than other experiments on the same animals with similar levels of heat production but with normal humidity. Between the limits of 40 to 80%, however, differences in humidity did not measurably influence the relation of insensible loss to heat production.

TABLE 1

Insensible loss of weight and heat production of rabbits[1]

Rabbit,[2] average weight, sex and date of experiment	Relative humidity	Per hour: Insensible loss	Per hour: Heat production
1939–40	%	*gm.*	*Cals.*
A, 2.20 kg. ♀			
Nov. 8	65	2.18	5.36
Nov. 17	66	2.53	5.16
Nov. 22	75	2.62	5.08
Nov. 29	71	2.43	5.23
Jan. 3	53	2.02	4.95
Jan. 30	53	2.19	5.13
Feb. 7	64	2.68	5.50
B, 2.23 kg. ♀			
Feb. 1	47	1.65	4.51
Feb. 5	47	1.73	4.61
C, 2.31 kg. ♂			
Oct. 13	75	2.16	4.91
Oct. 13	38	2.20	5.26
Oct. 25*	61	3.12	6.21
Oct. 26	59	1.96	5.43
Oct. 26	34	2.13	5.54
Oct. 31	50	2.12	4.78
Nov. 7	46	1.83	4.57
Nov. 15	49	1.70	4.70
Nov. 20	43	1.61	4.66
Dec. 27*	47	2.04	5.10
Dec. 28	43	1.57	4.67
Jan. 9	38	1.57	4.44
D, 2.40 kg. ♂			
Nov. 7	54	2.15	5.33
Nov. 16	57	2.16	5.57
Nov. 22	57	2.01	5.27
Dec. 1	45	1.50	4.77
Dec. 29	68	1.88	5.90
Dec. 29	38	2.03	5.79
Feb. 1	49	1.89	5.19
E, 3.37 kg. ♂			
Nov. 17	66	3.60	6.93
Nov. 25	79	4.17	7.94
Dec. 1	62	3.38	7.09
Jan. 4	67	4.13	7.72
Feb. 1	87	4.53	8.76
Feb. 8	66	4.72	7.81
F, 3.38 kg. ♀			
Oct. 10	52	2.48	5.98
Oct. 16*	58	2.80	6.65
Oct. 17	69	2.30	6.09
Oct. 17	40	2.54	6.33
Nov. 6	43	2.13	5.57
Nov. 14	53	2.30	5.84
Nov. 20	47	2.46	6.08
1939–40	%	*gm.*	*Cals.*
G, 3.57 kg. ♀			
Oct. 11	76	3.49	7.23
Nov. 7	76	3.75	7.60
Nov. 15	78	3.51	7.62
Nov. 23*	85	4.63	7.99
Nov. 24	74	3.96	7.23
Dec. 1	68	3.74	6.87
Dec. 21	63	4.25	7.18
Jan. 3	63	2.96	6.77
Feb. 7	54	4.26	6.97
H, 4.13 kg. ♀			
Nov. 16	85	4.56	9.22
Nov. 22	90	4.75	9.20
Nov. 29	95	5.18	9.49
Dec. 29	92	5.06	9.02
Feb. 2	81	4.10	8.02
Feb. 5	84	3.81	7.71
I, 4.24 kg. ♀			
Nov. 4	59	3.20	8.00
Nov. 9	76	3.94	8.41
Nov. 9	51	4.13	8.44
Nov. 16	61	3.63	8.60
Nov. 22	76	4.00	8.40
J, 4.31 kg. ♀			
Nov. 6	36	1.99	6.02
Nov. 10	38	1.90	6.10
Nov. 20	39	2.03	6.60
K, 4.83 kg. ♂			
Nov. 1	58	4.11	7.93
Nov. 7	71	3.91	8.41
Nov. 10	56	3.08	7.03
Nov. 21	65	3.38	7.27
Dec. 19*	72	5.02	9.19
Jan. 16	53	3.56	8.18
L, 4.94 kg. ♀			
Oct. 16*	71	4.44	10.00
Oct. 17	45	3.84	9.10
Nov. 9	71	3.66	8.67
Jan. 17	65	3.87	8.75

[1] Measured at 28° to 29°C., after fasting 24 hours at 28°C., except in those experiments marked with an asterisk, which were made immediately after food ingestion.

[2] The breed of rabbits A, B, C and D was Dutch, of rabbit E Chinchilla, and of all the others New Zealand White.

Fed versus fasting state. A few rabbits were measured immediately after eating and again 24 hours later, with no additional food intake. In these experiments the heat production was naturally greater on the first than on the second day. When these results were plotted and the values for the 2 days connected by a line, the slopes of these lines were consistently steeper than the average trend of all the data except in one instance, in which case the slope was the same as the average. Hence the relationship between insensible

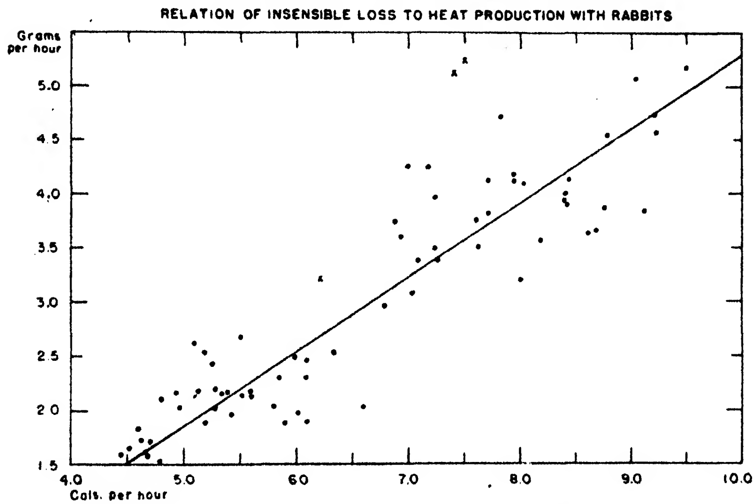

Fig. 1 Relationship between hourly insensible loss of weight and heat production per hour, simultaneously measured, with rabbits fasting 24 hours, at 28° to 29°C. The dots represent the values obtained at relative humidities between 34 and 95% and the crosses, the values obtained in experiments with very low humidity when calcium chloride was placed in the bottom of the respiration chamber.

loss and heat production was slightly different after food ingestion from that after 24 hours of fasting.

Body temperature. There was no consistent effect of differences in the normal level of body temperature, between 38.9° and 41.1°C., upon the relationship. The maximum variation in the body temperatures of the individual rabbit was frequently found when the relationship of the insensible loss of

weight to the heat production remained unchanged. There was also no tendency for the results obtained with rabbits having low or high average body temperature to be definitely below or above the average curve in figure 1. Hence no effect can be attributed to differences in normal body temperature.

Insensible loss as an index of heat production. Between the limits of 1.5 and 5.0 gm. of insensible loss per hour the straight-line relationship between this factor and the heat production of the adult rabbit, when post-absorptive, at 28° to 29°C., may be expressed by the equation

$$H = 1.45L + 2.32$$

in which H equals kg.-calories per hour and L equals grams of insensible loss of weight per hour. By this equation the heat production has been calculated from the measurements of the insensible loss, and the percentage deviations of the measured heat productions from these calculated or predicted heat values have been determined. In two-thirds of the sixty-eight experiments the individual percentage deviations for any given rabbit were within $\pm 5\%$ of its average percentage deviation. The standard deviation of the percentage differences for all the measurements is $\pm 8.9\%$. Measurements of the insensible loss of rabbits under the conditions set forth will serve for approximate calculation of the probable heat production prevailing at the time of such measurements but cannot replace accurate respiratory exchange measurements.

Percentage of heat loss in vaporization of water. The water lost by vaporization has been calculated for these experiments by correcting the insensible loss of weight for the carbon dioxide given off and the oxygen absorbed. From these results the percentages of heat lost through the path of water vapor have been calculated, on the basis that each gram of water vapor represents the loss of 0.576 kg.-calories. The ranges of these percentage losses in the individual experiments with the different rabbits and the average loss of each rabbit are shown in table 2. The average losses vary from 17.7 to 29.7% and average 24.6%. Computing similar values from the data of Mayer and Nichita ('29) obtained in experiments at environmental temperatures of 27° to 29°C., but with

rabbits not fasting, we find losses of 22.8, 24.5 and 27.2%. The two experiments of Marshall, Aydelotte, and Barbour ('31) at 30°C. with rabbits presumably fasting (R.Q. = 0.73 and 0.70) give 24.6 and 25.8%. Our data, therefore, support these fragmentary data. The percentages of heat lost by vaporization of water by other animals measured at thermic neutrality by the Nutrition Laboratory (Benedict, '36; Benedict and Lee, '36, '37 and '38; Ritzman and Benedict, '38) were: albino mice 18%, geese 22%, marmots 20%, cows 26% and an elephant 19%. The average value found by Newburgh et al. for man is 25%.

TABLE 2

Percentage of heat lost by rabbit in vaporization of water[1]

RABBIT	HEAT LOST IN WATER VAPOR		
	Minimum	Maximum	Average
	%	%	%
A	22.9	29.5	25.9
B	21.0	21.5	21.3
C	19.1	25.5	22.1
D	17.6	22.5	20.5
E	26.7	33.7	29.6
F	21.2	24.1	22.6
G	24.5	34.0	29.7
H	27.9	32.2	29.7
I	22.8	28.1	25.9
J	17.4	18.1	17.7
K	24.2	29.1	26.4
L	23.0	24.0	23.5
Average			24.6

[1] Calculated from data in table 1, as explained on page 302.

SUMMARY

Sixty-eight simultaneous measurements of the insensible loss of weight and the heat production of twelve adult rabbits, at 28° to 29°C., after fasting 24 hours at 28°C., showed that there was a relationship between these factors. This relationship was not measurably affected by variations in humidity in the range of 40 to 80%. Shortly after feeding, the relationship changed slightly. Fluctuations of the relationship could not be correlated with changes in normal body temperature.

Between the limits of 1.5 and 5.0 gm. of insensible loss per hour, the relationship was a straight-line function that can be expressed by the equation

$$H = 1.45L + 2.32$$

in which H equals kg.-calories per hour and L, grams of insensible loss per hour. The standard deviation of the percentage differences of the measured heat production from the heat production calculated from the insensible loss by this equation is $\pm 8.9\%$. Measurement of the rabbit's insensible loss of weight under the conditions set forth will give approximate values for the probable heat production prevailing at the time of measurement but will not replace exact measurements of the respiratory exchange. The heat lost by vaporization of water, as shown by indirect calculation, averaged 24.6% of the measured heat production.

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THE INTRAVENOUS ADMINISTRATION OF CRYSTALLINE AMINO ACIDS TO INFANTS [1]

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That crystalline amino acids may serve as the source of nitrogen for adequate nutrition of laboratory animals has been adequately proved by the long series of experiments initiated by Osborne and Mendel and brought to a successful conclusion by Rose and his collaborators. As far as we know such studies have never been extended to determine the adequacy of pure amino acids for human beings. The preparation of material for this type of experiment is costly and laborious; however, it is only by the use of such purified chemicals that the role of the amino acids in nutrition was determined. When native protein or protein degradation products are used, one is always confronted with the problem of whether the effects obtained are due to the amino acids or to some additional substance in the preparation used. Such was the case when Osborne and Mendel prepared diets from purified amino acids and found it necessary to include "protein-free milk", which carried not only essential minerals and vitamins, but also a small amount of nitrogen of undetermined origin.

Successful nutrition of both infants (Shohl et al., '39) and rats (Mueller et al., '40) has been reported, when the source of nitrogen in the diet consisted of hydrolyzed casein. That this product was free of native protein was attested by the fact that it was found suitable for intravenous use by a number of

[1] This study was supported in part by a grant from Mead Johnson & Company.

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investigators. Hydrochloric acid digests of casein are deficient in tryptophane and such preparations must be supplemented with this amino acid before positive nitrogen balances can be obtained (Elman, '39). An enzymatic hydrolysate was found to be a satisfactory source of nitrogen, since positive nitrogen balances were obtained when it was given intravenously with glucose and sodium chloride for short periods of time (Shohl et al., '39). The question of whether positive nitrogen balances in infants could be obtained as well by the use of crystalline amino acids became a matter of great interest.

It was reported that in a few instances of infants given intravenous injections of casein hydrolysate, the only untoward symptom observed was an increase in body temperature. The amount of amino acid administered was large, 2 to 3 gm./lb. of body weight per day (4 to 6 gm./kg.). Quantities of this order are required to obtain adequate positive balances; smaller amounts do not cause a rise in temperature. The question arose as to whether the hydrolyzed casein might contain a pyrogenic substance which could be eliminated during the preparation of the material or removed subsequently. If, on the other hand the property was inherent in the amino acids themselves as thus administered, then the increase in temperature was unavoidable. The most direct method of approach to this problem seemed to be to administer intravenously a mixture containing only crystalline amino acids.

The purpose of our investigation was to compare the effects of crystalline amino acids given intravenously with those of an enzymatic hydrolysate of casein, with regard to temperature reactions and nitrogen balance.

PREPARATION OF MATERIAL

The mixture of amino acids and the casein hydrolysate were supplied to us through the kindness of Dr. W. M. Cox, Jr.[2] The enzymatic hydrolysate of casein contains 11.9% of nitrogen, of which over 60% is present as amino nitrogen. The crystalline amino acid preparation was a modifica-

[2] Of Mead Johnson and Company.

tion of mixture XII of McCoy, Meyer and Rose ('35). The composition is given in table 1. It was necessary to reduce the amounts of tyrosine and cystine used because of their low solubilities. According to the findings of Rose ('38) cystine and

TABLE 1

Amino acid mixtures

	MIXTURE XII [1]	MIXTURE USED FOR INTRAVENOUS INJECTION
	gm.	*gm.*
glycine [2]	3.00	3.00
dl-alanine [2]	3.80	3.80
dl-valine [3]	16.00	16.00
l-leucine [3]	9.00	9.00
dl-isoleucine [2]	8.00	8.00
dl-norleucine [2]	2.50	2.50
l-proline [2]	8.00	8.00
l-hydroxyproline [2]	2.00	2.00
dl-phenylalanine [2]	7.80	7.80
d-glutamic acid [2]	22.00	22.00
d-aspartic acid [2]	4.10	4.10
dl-serine [3]	3.00	3.00
l-tyrosine [3]	6.50	2.00
l-cystine [3]	1.25	0.50
l-histidine HCl [3]	3.40	3.40
d-arginine HCl [3]	6.35	6.35
d-lysine diHCl [3]	11.55	11.55
l-tryptophane [3]	2.25	2.25
dl-methionine [2]	3.50	3.50
d-threonine [4]	—	2.50
$NaHCO_3$	12.86	12.86
	136.86	134.31

[1] McCoy, Meyer and Rose (J. Biol. Chem., vol. 112, p. 283, 1935–1936).
[2] Purchased from Eastman Kodak Company.
[3] Made and supplied by Dr. K. S. Kemmerer, Mead Johnson and Company.
[4] Purchased from Paul Lewis Laboratories, Milwaukee.

tyrosine are not essential amino acids, and the small amount of cystine is compensated in any event by the amount of methionine present. Rose found that threonine was a dietary essential for growth and it has therefore been added. When only the dl form of any acid was available, Rose used twice the amount

of the active form required, even though this might not have been necessary. The presence of hydrochloride in the histidine, arginine and lysine preparations required the inclusion of slightly larger amounts of these materials, in order to supply the requisite amount of amino acid. Rose calculated that 1.424 gm. of his mixture XII are equivalent to 1 gm. of "effective" amino acids. To obtain, therefore, "effective" amino acids equivalent to those of casein or the casein hydrolysate, it would be necessary to use about one-third more of the crystalline preparation. The pH of a 5% solution is 4.4 One hundred grams of the material were available.

The amino acid mixture was prepared for intravenous administration as follows. The dry mixture was dissolved in freshly distilled warm water. With the crystalline acids considerable frothing due to the evolution of CO_2 took place. NaCl and glucose were added, the solution made up to volume and filtered through an autoclaved Berkfeld filter. The water used was obtained the day the solution was to be given, from the general supply of the Hospital which was used for making all the solutions for intravenous use. Since these solutions did not give temperature reactions, they served as a control for the water used. The solutions were used the same day they were prepared. Culture showed them to be uniformly sterile. Because the sample of glucose used was apparently not completely free of nitrogen, the solution was analyzed for nitrogen after it was made.

The amounts of amino acids given as shown in table 2 are the net weights of the crystalline amino acid mixture (minus the $NaHCO_3$) and the nitrogen values are those found by analysis of the injected solution.

PROCEDURE

Preliminary studies were made of the effect on infants of intravenous administration of small amounts of the crystalline amino acid mixture. Observations were made upon six infants who were considered to be normal for the purposes of this study. The solution, prepared as above, contained 5% amino

TABLE 2

*Nitrogen metabolism**

INFANT	NO.	DATE	DURATION: Period	DURATION: Injection	NUTRIMENT: Type	NUTRIMENT: Volume	NUTRIMENT: Amino Acids	NUTRIMENT: Amino Acids	NUTRIMENT: Glucose	NUTRIMENT: Calories	NITROGEN: Intake	NITROGEN: Intake	NITROGEN: Urine	NITROGEN: Feces	NITROGEN: Balance	NITROGEN: Balance
			hrs.	*hrs.*		*cc.*	*gm.*	*%*	*%*	*per kg. per 24 hrs.*	*gm.*	*gm./kg./24 hrs.*	*gm./period*	*gm./period*	*gm./12 hrs.*	*gm./24 hrs.*
F.	1	11/22/39	24	6	hydrolysate	300	15.0	5.0	5.0	20	1.75	0.30	1.95	0.15		— 0.31
age	2	12/6–7/39	24	7	hydrolysate	335	16.7	5.0	5.0	23	2.00	0.34	2.24	0.41		— 0.65
3 mos.	3	12/8–9/39	24	9	hydrolysate	500	25.0	5.0	5.0	33	2.90	0.49	2.70	0.54		— 0.34
wt.	4	12/12–13/39	24	14	crystalline	500	25.0	5.0	5.0	33	2.92	0.49	2.98	0.34		— 0.40
5.9 kg.		*1st 12 hrs.*		*12*		*425*					*2.49*		*1.40*	*0.17* [2]	*+ 0.93*	*+ 1.86* [1]
		2nd 12 hrs.		*2*		*75*					*0.43*		*1.58*	*0.17* [2]	*— 1.32*	*— 2.64* [1]
H.	1	12/19–20/39	24	14	hydrolysate	745	25.0	3.3	6.7	65	2.98	0.66	2.78	0.27		— 0.07
age	2	12/27–28/39	24	15	crystalline	840	22.0	2.5	10.0	75	2.46	0.55	2.17	0.06		+ 0.23
2 mos.		*1st 12 hrs.*		*12*		*655*					*1.80*		*1.37*	*0.06*	*+ 0.37*	*+ 0.74* [1]
wt.		*2nd 12 hrs.*		*3*		*185*					*0.66*		*0.80*	*0*	*— 0.14*	*— 0.28* [1]
4.5 kg.	3	12/28–29/39	24		formula [3]	800				120	3.00	0.67	1.95	0.38		+ 0.67
	1	1/12–13/40	24	18	hydrolysate	920	23.0	2.5	7.5	80	2.80	0.60	2.45	0		+ 0.35
		1st 12 hrs.		*12*		*440*					*1.20*		*1.09*	*0*	*+ 0.11*	*+ 0.22* [1]
		2nd 12 hrs.		*6*		*430*					*1.60*		*1.36*	*0*	*+ 0.24*	*+ 0.48* [1]
D.	2	1/13–14/40	24		formula [3]	750					2.40	0.51	1.56	0.38		+ 0.46
age	3	1/16/40	12	12	crystalline	825	21.6	2.5	7.5	70 } 133	2.42	0.52 } 0.83	1.40	0.06	+ 0.96	+ 1.92 [1]
3 mos.	4	1/16–17/40	12		formula [4]	450				63	1.44	0.31	0.95	0	+ 0.49	+ 0.98 [1]
wt.	5	1/17/40	12		formula [4]	450				126 [1]	1.44	0.62 [1]	0.68	0.16	+ 0.60	+ 1.20 [1]
4.7–	6	1/22/40	12	12	NaCl +											
5.0 kg.					glucose	855	0		10.0	146 [1]	0		0.64	0.07	— 0.71	— 1.42 [1]
	7	1/22–23/40	12		formula [5]	300				84 [1]	0.96	0.41 [1]	0.32	0.15	+ 0.49	+ 0.98 [1]
	8	2/28/40	12	12	NaCl	510	0			0	0		0.88	0.13	— 1.01	— 2.02 [1]
	9	2/28–29/40	12		formula [5]	300				100 [1]	1.45	0.58 [1]	0.64	0.11	+ 0.70	+ 1.40 [1]

* The figures in italics represent subdivisions of the period, the figures for which are directly above.

[1] Calculated for 24 hours.

[2] The value for the two stools was divided arbitrarily.

[3] Five feedings in 24 hours.

[4] Three feedings in 12 hours.

[5] Two feedings in 12 hours.

acids, 5% glucose and 0.45% NaCl. It was given intravenously into a scalp vein, at a rate not usually exceeding 50 cc./hr.[3] At first only 5 cc. of this solution were given. As no febrile reactions or other untoward effects were observed, the amount was increased to 10 cc., then to 25, 50, 100 and 200 cc. Observations of rectal temperatures were made every 30 minutes. When given in these amounts, in no instance was there observed either a rise in temperature or other clinical deviation from normal, regardless of whether the solution was prepared as above or was sterilized by being autoclaved at 20 pounds pressure for 20 minutes before being filtered. We were then ready to increase the amount stepwise, to the point where all the nitrogen and caloric requirements of the infant could be met by material given intravenously.

When amounts greater than 15 gm. (300 cc. of 5% solution) of either the crystalline or hydrolyzed amino acid preparation were given to small infants, in the majority of cases a febrile reaction was observed. So far as we could determine, the temperature increases resulting from the crystalline acid mixture and the hydrolyzed casein mixture were alike. Therefore it was concluded that the effect was due to the amino acids themselves and not to an impurity or hydrolytic by-product in the casein hydrolysate. Further investigation of this problem will be reported elsewhere (Higgins, Shohl and Blackfan, '40).

Three normal white male infants were admitted to the hospital for the purpose of studying nitrogen metabolism. Parallel studies were made with casein hydrolysate and crystalline amino acid solutions on each infant so that the effects of the two materials could be compared. The amount of crystalline amino acid mixture was too limited to permit preliminary observations or lengthy collection periods. The infants were under observation on a constant diet of evaporated milk and corn syrup [4] with supplements of vitamins A, C and D for at least a week before the study was begun. During this fore-

[3] We are indebted to the House Staff of the Infants' Hospital, especially Dr. H. Shwachman for all the intravenous administrations.

[4] Karo.

period they gained in weight, satisfactorily. The general plan in studying the nitrogen metabolism was as follows. The last feeding of milk formula was given at 5 A. M. The infant was placed upon a metabolism bed, the intravenous administration started at about 9 to 10 A. M., and collections of excreta begun. The feces were marked with carmine. The urine was collected for separate 12-hour periods, and preserved with thymol chloroform. Analyses were made for volume, pH, NH_4, urea, amino acids, creatinine and total N of the urine, and of total N of the feces, according to the methods previously described (Shohl et al., '39).[5]

The fractions of N excreted in the urine are not detailed here because they so closely resembled the nitrogen distribution recorded in our former report (Shohl et al., '39). Compared with the findings after milk formula feeding, the amount of urinary amino acids was doubled and undetermined nitrogen was increased at the expense of urea. Examination of the urine showed absence of glucose or at most a trace. The feces following intravenous administration of amino acids were frequently lacking or were so small in amount that the nitrogen content was insignificant.

It was planned to give the solutions over periods of 24 hours, but this could not be carried out for two reasons: first, the flow could not be maintained at a uniform rate and, second, owing to the limitation of the total volume of fluid we were willing to give in a 24-hour period, the administration was necessarily completed at varying times short of 24 hours.

RESULTS

The observations on nitrogen intakes, outputs and balances are given in table 2.

The experiments were conducted consecutively, and the findings in each case led to modifications in subsequent procedure. Therefore the three cases are detailed separately.

In periods F1 and F2 the intakes of both nitrogen and calories were low. In F3 and F4 the nitrogen intake was

[5] We are indebted to Wilma Stanley Hill for most of the analyses and to Harriet O'Hara for the care of the infants in the Metabolism Ward.

higher, but the caloric intake only slightly increased. Under these conditions nitrogen equilibrium was not attained. The results obtained by equal amounts of the casein hydrolysate and the crystalline amino acid mixture (F3 and F4) were indistinguishable.

Realizing that the second 12-hour period was practically a starvation period, the urine for the two 12-hour periods was analyzed separately in F4. This clearly differentiates the first 12-hour period, which showed a considerable positive balance, from the second 12 hours which showed a considerable negative balance.

In the second infant, H., the caloric intake was augmented by increasing both the amount and the concentration of glucose in the solution. Although the amino acid concentration was lower, the total nitrogen per kilogram of body weight was higher than in F. Under these conditions, nitrogen equilibrium was attained for the whole 24-hour period with the casein hydrolysate (H1). Positive nitrogen balance was attained with crystalline amino acids (H2) although the nitrogen intake was lower than in H1, presumably because the caloric intake was significantly higher. Again as in F4, separation of the nitrogen output into two 12-hour periods shows a positive balance for the first 12 hours and negative balance for the second 12 hours, during 9 hours of which the infant fasted.

In the third infant, D, with a smaller amount of casein hydrolysate (D1) than in H1, and slightly increased caloric intake, positive balance for the 24 hours was attained. When this experiment is separated into 12-hour periods, it is shown that each short period gave a positive balance. In this experiment approximately equal amounts of nitrogen were given in the two 12-hour periods, although the rate was increased in the second 12-hour period. The 6-hour fast did not cause a negative nitrogen balance.

The crystalline amino acids were given (D3) more rapidly than in F4 and H2. The administration was completed in 12 hours. The nitrogen balance was positive and high. To obviate the effects of starvation, the infant was fed milk formula

during the second 12-hour period (D4) thus increasing both nitrogen and caloric intakes for the 24-hour period. This second 12-hour period also showed a positive nitrogen balance, as did a subsequent 12-hour formula period (D5).

When saline and glucose solution was given (D6) without amino acids, under exactly comparable conditions, nitrogen was lost at the rate of 1.4 gm. per day. When saline only (D8) was given, the rate of nitrogen loss was 2.0 gm. per day.

Observations were made on a period of milk formula feeding immediately following each amino acid administration after the period H2. The purpose was to determine whether the material given in intravenous administration and recorded as positive balance was subsequently excreted. If such had been the case, negative balances would have occurred in cases H3, D2 and D4. These periods, however, show positive balances of the order expected from the intakes. This is particularly well shown in D4 and D5, which not only show a normal percentage retention of nitrogen, but closely resemble each other.

The periods D7 and D9, following nitrogen starvation, demonstrate the avidity with which the body retains nitrogen to compensate for previous losses. In these two cases the amount of nitrogen excreted in the urine was actually smaller than when no nitrogen was given and the percentage of the nitrogen retained was raised to nearly half the amount consumed.

From these data it is concluded that crystalline amino acids and casein hydrolysate administered intravenously were equally well utilized by the body as shown by the fact that each resulted in retention of nitrogen, and that the retained nitrogen was not lost in the after-periods.

DISCUSSION

When the crystalline amino acid mixture and the casein hydrolysate were compared, approximately equal amounts of nitrogen were administered, although nearly one-fourth of the amino acids of the crystalline product were in the inactive form. It has been customary in nutrition experiments, when racemic amino acids are fed, to increase the amount of amino

acids to make up for the inactive material present. The inclusion of increased amounts of inactive amino acids reduces the proportion of some of the essential acids in the crystalline mixture. Therefore, when this mixture is used at low levels of intake, inadequacy of certain essential acids may result. At higher levels of intake the absolute amounts are sufficient regardless of proportion. However, some of the forms not occurring naturally in protein are apparently just as useful as the natural form, for growth. We desired to test the crystalline amino acid mixture under rigorous conditions, and therefore used amounts corresponding to the casein hydrolysate instead of larger amounts. It is of considerable interest, therefore, that the nitrogen balances are quite comparable for the two mixtures of amino acids. The nitrogen retentions with the casein hydrolysate and the crystalline mixture were so similar in parallel observations that they cannot be differentiated in these experiments.

Short periods of observation of nitrogen balance are valid only if the subject has attained a steady state under the conditions to be studied. The nitrogen retentions do not have quantitative validity unless the conditions have been constant for 5 to 7 days. If one goes from a high level of intake to a lower but still adequate one, there will be a transition period with negative balances or low retentions until positive balances are again established on the lower intake. Conversely when the intake is increased, temporary high positive balances result until the conditions again become stable. The fore-periods included ingestion of greater amounts of nitrogen (3.5 to 4.8 gm. N/day) than were given intravenously, so that the tests were made under conditions which were not favorable to positive balances.

The results with saline and glucose alone (D6) are a measure of the negative nitrogen balances which would have occurred had the injected nitrogen not been utilized by the body. These values could be used to correct the nitrogen balances obtained for the periods of fasting. No such calculations have been made, however, because qualitatively the meaning of the bal-

ances is clear without such computation, and the quantitative values have not sufficient validity to justify it. They do demonstrate, however, in a striking manner that the amino acids were utilized by the body and that they converted what would otherwise have been considerable nitrogen loss to large positive balances.

When food is given by mouth it is absorbed slowly and hence consecutive 12-hour periods do not show alternating plus and minus nitrogen balances. Amino acids administered intravenously must be utilized when presented and therefore their metabolism is more rapid than that of a corresponding amount of material given by mouth. This was demonstrated by the great variation in nitrogen excretion in successive 12-hour periods.

It may be argued that part of the amino acids given in any period was excreted in the following 12 hours. This would result in either a diminished positive balance or a negative balance in the second 12-hour period, as was the case in F4 and H2. This argument, however, does not take account of two other important factors, namely, the total calories given in 24 hours and the duration of fasting. When there is a long fast, (H2), nitrogen catabolism continues (as in D8) resulting in negative nitrogen balance. This rate of excretion is increased when fasting follows a period in which inadequate calories were given (F4). The conditions were most severe in F4 when a fast of 10 hours followed a period of inadequate caloric intake, less severe in H2 when more calories were supplied in the first 12 hours. When sufficient nourishment was given in the second 12-hour period even with a 6-hour fast (D1) negative balance was prevented. The most favorable results are found in D3 and D4, in which a high positive balance during intravenous administration was followed by positive balance in the second 12 hours, during which there was adequate caloric intake and no period of fasting.

Therefore, the negative balances in the second 12-hour period of F4 and H2 do not demonstrate delayed excretion and hence lack of utilization of ingested nitrogen, but taken in

conjunction with periods D1, D2, D3, D4, D6 and D8 do allow us to evaluate the excretion on the basis of the several factors involved. The conclusion is reached that for successful intravenous nutrition not only must adequate nitrogen and calories be supplied, but the administration should be continuous or at least without long periods of fasting.

The present studies confirm and extend our previous observations that, in conjunction with adequate fluid, glucose and sodium chloride, 0.5 gm. of N/kg./day given intravenously to a normal infant, will result in satisfactory nitrogen retentions for short periods of time.

SUMMARY

The rise in temperature which sometimes accompanies the intravenous administration of amino acids was found to be caused equally by casein hydrolysate and a crystalline amino acid mixture.

Positive nitrogen balances of the same degree were obtained by intravenous administration of similar amounts of a crystalline amino acid mixture and casein hydrolysate.

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FAT AS A FACTOR IN THE HEALING OF RICKETS WITH VITAMIN D [1, 2]

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ONE FIGURE

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The study of the effects of various factors on the efficiency of vitamin D action is very important. In the course of some studies in this laboratory, a diet of purified foodstuffs, low in lipids, was devised. This diet was modified so that it could be used for studies in experimental rickets, and a study of the influence of fat on the healing of rickets by vitamin D was undertaken.

McDougall ('38) reports that rats eating a diet containing a high proportion of wheat flour or bread, and low in calcium and vitamin D, were prevented from developing rickets by addition of 11% lard or olive oil. She ascribes these results to the fact that absorption of calcium is promoted by formation of calcium soaps, and in absence of fat, calcium combines with phytin (or its cleavage product) to form an insoluble compound which cannot be absorbed. Recent experiments of Boer ('39) have likewise indicated that rats on a rachitogenic diet are prevented from developing rickets when 10% margarine is added. This effect is ascribed to the fatty acids having an antirachitic action in the presence of small amounts of vitamin D; in other words, fats have a sparing action on vitamin D. Kon and Booth ('34 a, '34 b) are of a similar opinion.

[1] Presented before the American Chemical Society, Milwaukee, Wisconsin, September, 1938.

[2] This work was aided by a grant from the Winthrop Chemical Company.

EXPERIMENTAL

Young piebald rats, weighing from 45 to 60 gm., were used as experimental animals and were placed on the rachitogenic diet at about 25 days of age. At the end of the 21 days, when a uniform degree of rickets had developed as evidenced by x-ray pictures, the animals were then ready for the test period. As a basis of procedure for determining the efficiency of vitamin D, we followed essentially the method for assay of vitamin D in cod liver oil, as described in the U. S. Pharmacopeia XI. The test period was 10 days, and on the eleventh day, examination for the degree of healing was judged by both the x-ray and the line test. The degree of healing by the line test was indicated by the following conventional rating used in this laboratory (Knudson and Benford, '38): 1+ indicates a broken line of calcified bone; 2+, a narrow continuous line; 3+, a broad continuous line; 4+, practically complete calcification.

The percentage composition of the rachitogenic diet (6C) is as follows: lactalbumin 7HAAX [3] 18, sucrose 72, salt mixture 2, rice polish concentrate [3] 5, calcium carbonate 3. To each kilogram of this food mixture are added 2 mg. of riboflavin dissolved in alcohol, and 20 mg. of carotene dissolved in ether. The calcium and phosphorus contents were 1.27 and 0.25%, respectively; the Ca to P ratio was 5 to 1.

The salt mixture used in the above diet is a modification of the Osborne and Mendel salt mixture no. XXX ('26–'27), free from calcium and phosphorus, and used by them in rachitogenic diets. It is simplified from the Osborne and Mendel salt mixture in that only solid ingredients are used. Its composition (in grams) is as follows: magnesium citrate 31.0, magnesium sulfate 11.3, potassium chloride 128.0, sodium chloride 33.2, ferric citrate ($1\frac{1}{2}$ H_2O) 6.34, manganese sulfate ($2H_2O$) 0.02, sodium fluoride 0.248, and potassium aluminium sulfate ($K_2Al_2(SO_4)_4$, 24 H_2O) 0.0538.

The lactalbumin, 7HAAX, is a highly purified preparation, free from lipid material, calcium, and phosphorus. Rice polish

[3] Obtained from The Borden Company, New York.

concentrate contains from 0.4 to 0.9% lipid material, and 4.6% of phosphorus. The phosphorus content of the diet is therefore derived practically entirely from the rice polish. The total lipid content of the diet is less than 0.05% and this is derived from the rice polish concentrate. The calcium and

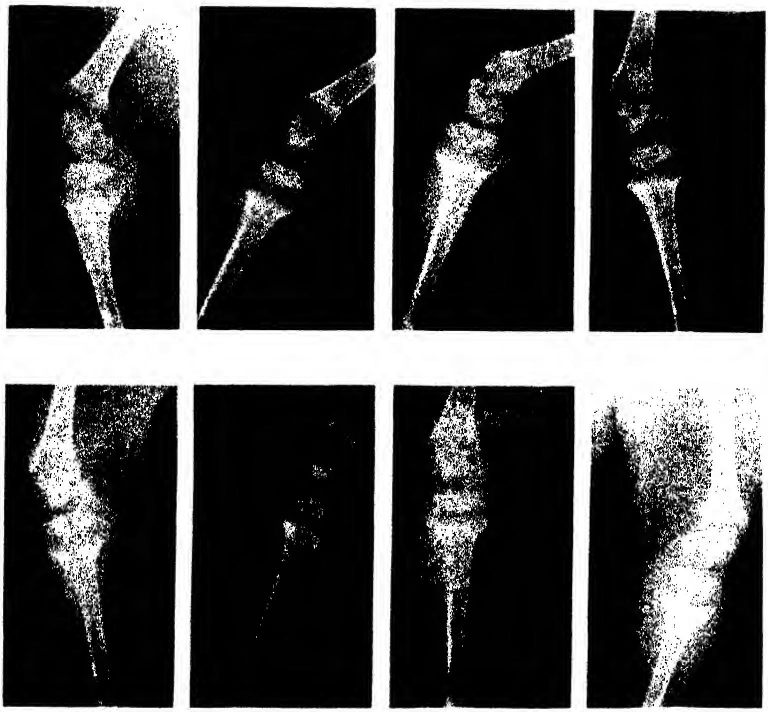

Fig. 1 The upper row shows the x-ray photographs of knee points of rats at the end of 3 weeks' subsistence on the 6C diet. The lower row shows the knee joints of rats at the end of 3 weeks on the Steenbock-Black 2965 diet.

phosphorus contents of the above diet are about the same as those for the Steenbock-Black diet 2965 ('25). The only unpurified material in diet 6C is the rice polish concentrate.

A marked uniform degree of rickets is produced with this ration, which is similar to the degree of rickets obtained with the Steenbock-Black 2965 diet. In figure 1 are presented x-ray

photographs of knee joints of rats showing the development of rickets on these two diets at the end of 3 weeks.

The ash contents of fat-free, dry extracted femurs of rats on the 6C diet, and Steenbock-Black diet, for 21 days, are also very similar. The average of ten rats on the 6C diet showed an ash content of 28.9%, which may be compared with 29.9% for the Steenbock-Black 2965 diet. The healing effects produced by vitamin D when given to rats on these two diets also show excellent agreement. There were twenty rats on each test dose with each of the diets and the results are given in table 1.

TABLE 1

Comparison of healing of rickets in rats subsisting on 6C diet and Steenbock-Black diet 2965

DATE	MATERIAL TESTED	DEGREE OF HEALING	
		6C diet	Steenbock-Black diet 2965
2-12-38	35 mg. U. S. P. reference cod liver oil	3.1+	2.8+
2-21-38	35 mg. U. S. P. reference cod liver oil	2.9+	2.7+
2-10-38	0.08 μg. vitamin D_2 in propylene glycol	2.5+	2.5+
2-12-38	0.10 μg. vitamin D_2 in propylene glycol	3.2+	3.1+
2-18-38	0.08 μg. vitamin D_3 in propylene glycol	3.1+	3.4+
2-21-38	0.10 μg. vitamin D_3 in propylene glycol	3.5+	3.3+

To study the effect of fat on the healing of rickets by vitamin D, 5, 10 or 20% of a hydrogenated fat [4] was substituted for a corresponding amount of sucrose in the 6C diet. For comparison, similar amounts of fat were substituted for gluten flour in the Steenbock-Black 2965 diet. The results of this study are presented in table 2.

In the first series of experiments, 0.025 μg. of crystalline vitamin D_2 [5] dissolved in propylene glycol, was added to 40 gm. of diet. This amount of diet was consumed in all cases within 6 to 7 days. It will be noted that the healing is about three times greater with 5% of fat in the diet than with no fat. With 10% of fat, the healing is markedly lessened, and with 20%

[4] Crisco.

[5] We are grateful to Dr. O. W. Barlow of the Winthrop Chemical Company for supplying the crystalline vitamin D_2.

of fat, even more so. The Steenbock-Black 2965 diet itself contains 2.75% of total lipids, so that in these experiments slightly more fat was present than in those on the 6C diet. In the second series of experiments, the same amount of vitamin D_2 was used except that it was given by mouth in four doses on alternate days, i.e., on the first, third, fifth, and seventh

TABLE 2

Effect of fat on the healing of rickets

FAT IN DIET	6C DIET		STEENBOCK-BLACK 2965 DIET	
	Number of rats	Healing	Number of rats	Healing
Series I. 0.025 μg. vitamin D_2 in propylene glycol added to 40 gm. diet				
No fat added	10	0.6+	10	0.6+
5%	10	2.2+	10	1.7+
10%	10	1.6+	10	0.9+
20%	10	1.2+	10	0.7+
Series II. 0.025 μg. vitamin D_2 in propylene glycol given separately in four doses on alternate days (1, 3, 5, 7 day)				
No fat	10	0.9+	10	0.8+
5%	10	2.0+	10	1.1+
10%	10	1.7+	10	0.8+
20%	10	1.3+	..	....
Series III. 0.1 μg. vitamin D_2 dissolved in propylene glycol given separately in four doses on alternate days (1, 3, 5, 7 day)				
No fat	20	1.45+	20	1.4+
3%	10	1.55+	..	....
5%	20	2.25+	20	2.3+
10%	20	1.2 +	20	1.8+

days. While the results are not quite so striking, nevertheless, they do indicate that 5% of fat added to the diet is more effective than 10 or 20% in bringing about healing of rickets. In the third series of experiments 0.1 μg. of vitamin D_2 was given separately in four doses by mouth and a similar result was obtained with 5% of fat added to the diet. In one of the experiments in this series on the 6C diet, only 3% of fat was present and the healing was practically no greater than with no fat.

In all of these experiments, whether vitamin D was given by mouth or separately, 5% of fat in the diet brought about much greater healing than with no fat. Larger amounts of fat in the diet, such as 10 or 20%, produced progressively less healing, although the healing was generally greater than with no fat.

DISCUSSION

The fact that 5% of fat in the rickets diet produces a much better healing with a known amount of vitamin D than does a diet with 10 or 20% of fat, is very significant. One may well ask how this smaller amount of fat produces its effect, and why a larger amount of fat should be less effective. Ten and 20% of fat in the diet are by no means to be considered excessive. McDougall ('38) expressed the view that the rachitogenic action of cereals is due to a deficiency of fat, and that a normal amount of fat seems to be important for the solubility in the intestine of calcium and phosphorus. She points out that the fatty acids produced by hydrolysis form calcium soaps which are then rendered soluble by the action of bile and thus readily absorbed. That the formation of calcium soaps in the presence of fat does have some influence on the healing of rickets obtained with vitamin D seems evident from a series of experiments in which the calcium carbonate in the 6C diet was replaced by an equivalent amount of calcium in the form of calcium oleate, or calcium stearate. The fatty acid combined with calcium in this diet was about equivalent to the total fatty acids that would be derived from the hydrolysis of 20% of fat in the diet. In another series of experiments, one-fourth of the calcium carbonate was replaced by an equivalent amount of calcium in the form of an oleate or stearate. This would be equivalent to the fatty acids obtained with 5% of fat in the diet. The results of these tests are given in table 3.

It is to be noted that when all of the calcium is present in the form of calcium oleate or stearate or an equivalent mixture of both, there is considerably more healing than with calcium in the form of a carbonate. There is, however, no significant

difference between healing obtained with the calcium in the form of an oleate or a stearate. When only one-fourth of the calcium carbonate is replaced by an equivalent amount of calcium in the form of an oleate or stearate, the healing is about the same as with calcium carbonate. These results are extremely interesting, and rather surprising. In the series with calcium soaps containing combined fatty acids equivalent to the fatty acids obtained from the hydrolysis of 5% of fat

TABLE 3

Effect on the healing of rickets by vitamin D of replacement in the 6C diet of calcium in the form of a carbonate by calcium in form of a soap

FORM OF CALCIUM IN DIET	NUMBER OF RATS	DEGREE OF HEALING
	0.025 μg. vitamin D given separately in 4 doses	
Control on 6C diet	10	1.2+
¼ $CaCO_3$ replaced by an equivalent amount of Ca in the form of stearate	10	1.5+
All $CaCO_3$ replaced by an equivalent amount of Ca in the form of stearate	10	2.3+
¼ $CaCO_3$ replaced by an equivalent amount of Ca in the form of oleate	10	1.4+
All $CaCO_3$ replaced by an equivalent amount of Ca in the form of oleate	10	2.0+
¼ $CaCO_3$ replaced by an equivalent amount of Ca in the form of a mixture of equal parts oleate and stearate	10	1.2+
All $CaCO_3$ replaced by an equivalent amount of Ca in the form of a mixture of equal parts oleate and stearate	10	2.1+

in the diet, there is no more healing than with the fat-free controls, which is in marked contrast to the greatly increased healing obtained with 5% of fat in the diet. However, in the series with the calcium soaps containing combined fatty acids equivalent to the fatty acids obtained from 20% of fat in the diet, the healing is much greater than with 20% of fat in the diet. The results of these tests with calcium soaps would seem to contradict those given in table 2 with added amounts of fat to the diet. In view of these apparently contradictory

results it seems evident that the greater effectiveness of 5% of fat in the diet over 20% of fat in the diet cannot be explained on the basis of the formation of calcium soaps.

Boyd, Crum and Lyman ('32) have indicated that the presence of a moderate amount of fat in the diet may have a beneficial influence on absorption of calcium and phosphorus by maintaining a favorable acidity of the intestinal contents. On the other hand, Nicolaysen ('38) did not obtain any greater utilization of calcium on a diet with 20% of fat than when no fat was added to a fat-poor ration consisting of corn, wheat-gluten, sodium chloride, and calcium carbonate. Zucker and Barnett ('23) explain the influence of fat on the prevention of rickets as due to the formation of insoluble calcium soaps, and phosphate in soluble form which would otherwise be precipitated as calcium phosphate, is made available for absorption.

These various viewpoints do not offer us an explanation for the fact that 5% fat is more effective than 10 or 20% of fat in the healing of rickets with vitamin D. It may be possible that more than one factor is concerned with this interesting phenomenon and that before any explanation can be given, more information than is covered in our experiments or those of others, will have to be obtained.

SUMMARY

1. A rachitogenic diet has been devised which is made up largely of purified materials and which is low in lipids.

2. Addition of 5% of fat to the rachitogenic diet gave a much better healing with a known amount of vitamin D than with no fat in the diet.

3. With the addition of 10 or 20% of fat, the healing of rickets by vitamin D was less than with 5% of fat, but greater than was obtained with no fat.

4. Replacement of all the calcium carbonate in the 6C diet by an equivalent amount of calcium in the form of an oleate or stearate, resulted in better healing. Replacement of only

one-fourth of the calcium carbonate by an equivalent amount of calcium in the form of an oleate or stearate did not result in any appreciable difference in the healing with vitamin D.

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THE DETERMINATION OF ASCORBIC ACID IN EVAPORATED MILK, POWDERED MILK AND POWDERED MILK PRODUCTS [1]

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Within recent years evaporated and powdered milks have received some consideration as a potential source of vitamin C. This problem has apparently received little attention in America.

Schlemmer, Bleyer and Cahnmann ('32), Henriksen ('37), Palladina and Anoshkina ('37), Taniguti, Hamamoto, Hirata and Suzuki ('37), Tomoi and Tomita ('37), Henry and Kon ('38), Kon ('38) and Henry, Houston, Kon and Osborne ('39) have studied the ascorbic acid content of different milk preparations. Phospho-18-tungstic acid and various modifications of the indophenol titrimetric technique were used by them. In most cases the work on the milk preparations themselves was not verified by biological assay nor was any evidence presented as to the specificity of the reaction involved in the analysis.

Further study of the photoelectric method suggested by Woessner, Elvehjem and Schuette ('39) has disclosed certain improvements especially valuable when determining dehydroascorbic acid. We wish therefore to describe the improved method as applied to evaporated milk, powdered milk, and

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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powdered milk products and to cite experimental evidence that the method is indeed specific for ascorbic acid.

EXPERIMENTAL PART

The samples of evaporated milk were prepared by diluting one part of the evaporated milk with one part of copper-free water. Samples of powdered milks and powdered milk products were prepared by dissolving 145 gm. of the powder in 1 liter of copper-free water. Heating to 50°C. was necessary in order to bring the roller-dried products into solution. All powders were stirred into the water with an ordinary silver-plated spoon.

The samples were analyzed for both the reduced and reversibly oxidized ascorbic acid. The reduced ascorbic acid was determined according to the procedure already described by the authors ('39). However a very slow sliding of the galvanometer is always observed with evaporated milk. Thus it is necessary to take the galvanometer reading not later than 15 seconds after the indophenol has been added to the colorimeter tube if the true reduced ascorbic acid is to be determined accurately.

The procedure for the determination of the dehydroascorbic acid has been modified as follows: After the addition of a few drops of dibutyl phthalate to the milk in order to prevent foaming, wet hydrogen sulfide is bubbled through the milk for exactly 20 minutes. Then as rapidly as manipulation will permit, 25 ml. of the hydrogen-sulfide-saturated milk are added to 75 ml. of modified Willberg reagent and the whole shaken well to break the resulting curd into small particles. The hydrogen sulfide is removed immediately by passing a vigorous stream of wet oxygen-free nitrogen through the suspension. Our experience has shown that this operation takes no longer than 20 minutes for 50 ml. of the curd-reagent mixture. After the curd is removed by filtration, 5 ml. of the filtrate are measured into one of the colorimeter tubes. We have never experienced difficulty in obtaining a crystal-clear serum at this point. Then with the simultaneous start of a stop watch

10 ml. of the dye-acetate solution are added to the tube by means of a rapidly delivering pipette and the galvanometer readings at 15 and 30 seconds are recorded. The galvanometer reading corresponding to zero seconds, for practical purposes, can be considered equal to the difference of the galvanometer readings at 15 and 30 seconds subtracted from the galvanometer reading at 15 seconds.

The calculations and other details of this modified procedure are identical with those described in the original paper. This modified procedure is also recommended for use with whole milks, raw and pasteurized, as it gives more reliable results especially when determining dehydroascorbic acid.

DISCUSSION

Experiments conducted with dehydroascorbic acid solutions in 10% aqueous sodium chloride prepared either by iodine oxidation or use of ascorbic acid oxidase of Tauber, Kleiner and Mishkind ('35) showed that at hydrogen ion concentrations comparable to those of fresh milk and milk preparations recovery of dehydroascorbic acid was quantitatively complete when hydrogen sulfide was introduced for 20 minutes or longer. It was not possible to obtain 100% recovery at a pH 2 or lower even after 8 hours of hydrogen sulfide treatment. This is in accordance with the experiences of Bessey ('38) but not in agreement with the observation of Kon and Watson ('36).

Like de Haas and Meulemans ('36), we have observed that hydrogen sulfide treatment of milk reduces other substances besides dehydroascorbic acid. Since these reduced substances react with the indophenol at a slower rate than ascorbic acid at the final pH used in the colorimeter tube, the interference can be eliminated by extrapolation to zero time. However if the hydrogen sulfide treatment is extended much beyond 20 minutes, it becomes increasingly difficult to extrapolate with acceptable accuracy.

Heated lactose solutions (pH 6) introduce no error. However when heated lactose solutions were treated with hydrogen

sulfide under the conditions of the experiment a very slow movement of the galvanometer was observed. The interference was not serious and could be eliminated by extrapolation to zero time.

Redissolved washed casein did not contribute to the formation of interfering substances upon hydrogen sulfide treatment. It was found that at the pH of milk riboflavin offered no interference as it did with the original method at the higher hydrogen ion concentrations. Hydrogen sulfide treatment of lactoglobulin solutions prepared from evaporated milks produced a marked interference very similar to that observed under the conditions of the procedure suggested. This interference also could be eliminated by extrapolation to zero time. The lactalbumin fraction of milk also indicated formation of interfering substances on treatment with hydrogen sulfide but to a much less marked degree. This interference was likewise successfully eliminated by extrapolation.

The fact that these effects were more marked with the more drastically heated milk products than with the raw or pasteurized milks, seems to indicate that reducible soluble proteins or protein fractions have been formed during the processing. It is very likely also that some of these reducible substances are sulfhydryl compounds. Josephson and Doan ('39) reported that sulfhydryl compounds are formed in milk if it is heated to a sufficiently high temperature. Their observation that these compounds are readily oxidized with copper is in agreement with the observations made in this laboratory. They suggest that the lactalbumin of the milk is the most likely ingredient responsible for this condition and state that the protein of the fat globule adsorption membrane may also be involved.

Since qualitative examination of a white flocculent precipitate obtained from a strongly ammoniacal evaporated milk serum gave a positive test for tin and a very faint test for iron, the reaction of these metals in the procedure was studied. Stannic and stannous tin solutions did not accelerate the oxidation of ascorbic acid under semi-anaerobic conditions.

Stannic tin itself does not interfere although when treated with hydrogen sulfide it contributed to the formation of stannous tin which partially remains in solution under the conditions of the method. At the final pH in the colorimeter tube (4.1) the stannous tin reacts at a slower rate than the ascorbic acid. Hence no error is introduced if the readings are extrapolated to zero time.

The case of iron is more serious. Ferric iron does not react with 2,6-dichlorophenolindophenol but the hydrogen sulfide treatment always produces some ferrous iron which remains in solution. The ferrous iron reacts instantaneously with the indophenol at the final pH of the solution in the colorimeter tube. Since milk picks up, at most, an insignificant quantity of iron from the processing which it undergoes, the error introduced from this source under ordinary conditions is nil. A sample of evaporated milk, by analysis found to contain a total of 15 mg. of ascorbic acid per liter, was treated for 15 minutes with the ascorbic acid oxidase of Tauber, Kleiner and Mishkind ('35). Analysis then showed no reduced ascorbic acid present but treatment with hydrogen sulfide for 20 minutes indicated 15 mg. of dehydroascorbic acid per liter. If the oxidase-treated milk was heated to 100°C. for 10 minutes, the analysis showed no ascorbic acid. However if either copper, hydrogen peroxide or intense irradiation was used to destroy completely the ascorbic acid present, analyses would always indicate reducing activity following hydrogen sulfide treatment. Since no reducing activity was ever obtained after hydrogen sulfide treatment, when use was made of ascorbic acid oxidase followed by heat, it is deemed that a non-specific oxidizing procedure, besides causing the irreversible destruction of ascorbic acid, will also cause the formation of substances which, when reduced with hydrogen sulfide, are capable of instantaneous reaction with indophenol at pH 4.1. If iron had been present in sufficient quantity to interfere, the oxidase-treated heated sample would have given a positive value for dehydroascorbic acid. Furthermore, tests on the serum of the milk before and after hydrogen sulfide treatment

failed to give a positive reaction for iron. The value of the ascorbic acid oxidase in deciding whether or not all reducing activity is due to ascorbic acid is only valid if interference due to stannous tin and reduced proteins can be eliminated, for we have observed that ferrous iron and stannous tin are oxidized by ascorbic acid oxidase. Since copper also catalyzes these reactions, this may be evidence in support of the essential constituent of the enzyme.

Further assurance that the method is specific for ascorbic acid was given by biological assay of samples of evaporated milk which showed wide variance in their ascorbic acid content. The same samples showed similar potency by biological and chemical assay.

Results of the determinations by method presented. The results compiled in tables 1 and 2 were secured in the normal process of testing the method under working conditions. Since it was not possible to obtain information as to the age of the samples, the time of the year they were prepared or other pertinent data, they are included here to give an indication of the ascorbic acid content of powdered and evaporated milks as purchased throughout bakery, grocery, and drug stores in a city of moderate size. A few general conclusions may also be drawn from the data.

It is apparent from tables 1 and 2 that the ascorbic acid content of powdered milk is roughly equal to that of evaporated milks. The powdered milk products, however (items 8 and 9 in table 1 for example), are for the most part significantly inferior to powdered milks and the average evaporated milk. It appeared that neither the spray process, the roller process, nor even the mode of storage, is the significant factor which determines the ascorbic acid content of the powder. It is possible that, unless special precautions are used, a greater quantity of heat-labile dehydroascorbic acid is formed the more the powdered milk and powdered milk products are handled or modified. Thus, when the dehydration is brought about by heat, the destruction of the antiscorbutic potency is in direct proportion to the quantity of dehydroascorbic acid

TABLE 1

Ascorbic acid content of powdered milks and powdered milk products

MATERIAL TESTED	ASCORBIC ACID PER LITER[1]			
	Reduced	Oxidized	Total	Per kg. of powder
	mg.	*mg.*	*mg.*	*mg.*
1. Open barrel, skim, spray	9.0	2.4	11.5	78.8
2. Open barrel, skim, spray	1.3	1.2	2.6	17.9
3. Closed tin, skim, roller	7.2	1.8	9.1	62.7
4. Open barrel, whole, roller	9.6	4.4	14.0	97.1
5. Vacuum, whole, spray	9.4	1.2	10.6	68.6
6. Spray dried, modified by addition of milk fat and sugar. Vacuum	7.9	0.4	8.3	57.9
7. Superior quality milk, part of fat removed, fortified by rice extract; irradiated and dried by roller process, vacuum	13.4	1.2	14.6	100.8
8. Protein milk, high in protein, lactic acid and low in lactose, pH 4.8, vacuum	2.6	4.3	6.9	48.0
9. Soluble casein, lactalbumin, lactose, milk salts, dextrins, maltose, extracts of yeast and wheat embryo. Vacuum	1.8	0.0	1.8	12.6

[1] Powder was dissolved to give a milk containing 12.5% of total solids.

TABLE 2

Ascorbic acid content of commercial evaporated milk

PRODUCT TESTED	ASCORBIC ACID PER LITER[1]		
	Reduced	Oxidized	Total
	mg.	*mg.*	*mg.*
Brand 1	3.0	0.8	3.8
Brand 2	3.9	1.5	5.4
Brand 1	4.6	2.4	7.0
Brand 3	4.9	4.7	9.6
Brand 4	8.0	1.6	9.6
Brand 5	11.2	1.5	12.7
Brand 6	11.8	1.9	13.7
Brand 7	10.0	4.1	14.1
Brand 8 Irradiated	19.5	2.8	22.3
Brand 8 Irradiated	20.1	0.3	20.4
Brand 9 Zuker Process	13.7	5.7	19.5
Brand 10 Irradiated	7.8	0.1	7.9
Brand 11 Irradiated	1.7	5.7	7.4
Brand 10 Irradiated	6.5	0.2	6.7
Evaporated goats' milk	14.8	0.0	14.8

[1] As milk comes from can, undiluted. Divide by two in order to convert to milligrams per liter of normal whole milk.

present. Consequently it appears that powdered milks are potentially a significant source of antiscorbutic factor if they are prepared from a milk which is rich in reduced ascorbic acid at the time of manufacture.

We have observed that milk can be aerated at 100°C. for 5 minutes with small loss of ascorbic acid. Consequently it is not surprising to find that a milk powder dissolved at 20°C. or at 50°C. will yield milks which show no detectable difference in their ascorbic acid content if the milk prepared at the higher temperature is cooled soon after its preparation.

The data of table 2 reveal that the ascorbic acid content of irradiated evaporated milks is not significantly different from that of the non-irradiated. This is understandable in view of the observation made by Scheer ('39) that only a slight reduction in ascorbic acid occurs when milk is irradiated with the intent of fortifying it with vitamin D and that this reduction is without practical significance.

The Evaporated Milk Association cooperated with this investigation in the collection of twenty-one samples of evaporated milk manufactured during the first week of April, 1940, in various plants throughout this country. The analyses of these samples are given in table 3. The sterilization temperature was 242° F. for 14 minutes and the forewarming or preheating temperatures varied from 204° F. to 212° F. Analyses were completed 12 to 16 days after the samples were manufactured.

The data reveal that the metallic nature of the manufacturing equipment is not the limiting factor which determines the ascorbic acid content of evaporated milk. This is proved by the wide variance in ascorbic acid shown by milks manufactured in equipment consisting of various combinations of copper, tinned copper and stainless steel. Evaporated milks manufactured almost entirely in copper equipment may contain more ascorbic acid than some evaporated milks manufactured almost entirely in stainless steel equipment.

The survival of any ascorbic acid at all while the milk is being subjected to evaporation in a copper pan may be ex-

plained by the presence of sulfides which are formed when the milk is heated, as well as a scarcity of oxygen. The sulfides possibly combine with any trace of copper which may dissolve and also act as reducing agents during the process. The latter point is not entirely a speculation in the light of observations by Sharp, Trout and Guthrie ('36) and Josephson and Doan ('39) who showed that ascorbic acid is protected to a considerable degree when the milk is heated to sufficiently high temperatures even when small amounts of copper are present. Josephson and Doan ('39) postulate that this action

TABLE 3

Ascorbic acid content of evaporated milks[1]

LOCATION OF PLANT	MATERIAL OF CONSTRUCTION[2]			ASCORBIC ACID PER LITER[3]		
	Pre-heater	"Hot wells"	Evaporating pan	Reduced	Oxidized	Total
				mg.	mg.	mg.
Colorado	none	Sn-Cu	Cu	24.0	3.6	27.6
Idaho	none	Ni	Cu	13.8	2.0	15.8
Illinois	Cu	S.S.	Cu	13.3	2.5	15.8
Indiana	S.S.	S.S., Cu	S.S.	15.8	0.0	15.8
Iowa	S.S.	Sn-Cu	Cu	12.0	0.0	12.0
Kentucky	Sn-Cu	Ni	Cu	7.2	0.8	8.0
Maryland	S.S.	Cu	S.S.	5.0	1.2	6.2
Michigan	S.S.	S.S., Cu	S.S.	6.2	1.8	8.0
Minnesota	Cu	Sn-Cu	Sn-Cu, S.S.	22.4	1.6	24.0
Mississippi	none	Ni	Cu	7.2	2.8	10.0
Missouri	S.S.	S.S. or Cu-Ni alloy	S.S.	11.6	2.2	13.8
Ohio	S.S.	Sn-Cu	Cu	11.6	2.2	13.8
Oregon	S.S.	Sn-Cu, S.S.	Cu	10.0	3.8	13.8
Tennessee	S.S.	Ni	Cu	5.0	3.0	8.0
Texas	Sn-Cu	Sn-Cu	Cu	4.6	2.6	7.2
Utah	none	Ni	Cu	20.8	0.0	20.8
Virginia	S.S.	S.S.	Cu	6.7	3.3	10.0
Washington	S.S.	Sn-Cu	Cu	4.0	0.0	4.0
Wisconsin	S.S.	S.S.	Cu	17.6	1.6	19.2
Wisconsin	none	Cu	Cu	14.8	1.0	15.8
Wisconsin	Cu	glass	Cu	7.7	0.8	8.5
			Average	11.5	1.7	13.2

[1] Supplied by the Evaporated Milk Association.

[2] Sn-Cu, tinned copper; S.S., stainless steel.

[3] As milk comes from the can, undiluted. Divide by two in order to convert to milligrams per liter of normal whole milk.

is due to the formation of sulfhydryl compounds in the milk at the elevated temperatures.

We have observed that its dehydroascorbic acid is completely destroyed when whole milk, containing about equal quantities of ascorbic and dehydroascorbic acids, is heated for only 10 minutes at 100°C. Thus it is understandable that the dehydroascorbic acid content of evaporated milk is insignificant and for practical purposes its determination could be omitted. The very small quantity of dehydroascorbic acid found indicates that this acid is completely destroyed during the evaporation and sterilization of the milk. Apparently sulfhydryl compounds or sulfides cannot inhibit the thermal rupture of dehydroascorbic acid. Consequently the actual quantity of ascorbic acid which survives during the processing is largely dependent on the quantity of the acid in the reduced form in the milk as it is received before concentration.

In attempting to prevent ascorbic acid losses it is apparent, from table 3, that either the omission or inclusion of a preheating step in the process of manufacture is not important. Likewise neither the quality nor the quantity of the stabilizing salts used exerts any influence here. Moreover the ascorbic acid content cannot be correlated with geographical site of production of the evaporated milk.

If the values cited for commercial evaporated milks in tables 2 and 3 are divided by two in order to express them on the basis of whole milk, we find that the average American evaporated milk supplies 25% of the quantity of ascorbic acid found in freshly drawn cow's milk. It should be emphasized, also, that this observation is based upon the analyses of evaporated milk samples which were fresh, that is, stored for no longer than 2 weeks. That a different picture may be obtained after the evaporated milks have been stored for a longer period, is quite probable inasmuch as Henry, Houston, Kon and Osborne ('39) observed that ascorbic acid is lost slowly but steadily under such conditions. We have a range, however, of 1.9 to 13.8 mg. of ascorbic acid per liter. This range indicates that evaporated milk is potentially a signifi-

cant source of ascorbic acid. Raw milk contains on an average 22.6 mg. of ascorbic acid per liter. This demonstrates that in certain of the samples of evaporated milk only about a 50% loss of ascorbic potency has occurred. The maximum value of 13.8 mg. per liter is not much lower than the average value of 17.3 mg. per liter, which we have reported for commercial raw milk, nor is it significantly different from the average value of 12.6 mg. per liter which we have reported for commercial pasteurized milk.

SUMMARY

The improvements of the method previously suggested by the authors make the method less time-consuming and the determination of dehydroascorbic acid more reliable. Among the substances found to make the use of the photoelectric colorimeter essential are heated lactose, stannous tin and certain of the water soluble proteins or protein fractions. The reliability of the photoelectric colorimeter is limited only by iron which fortunately is not present in significant quantities in milk or milk products.

The analyses of forty-one evaporated milks and nine powdered milks are given. The data reveal that the metallic nature of the manufacturing equipment is not the limiting factor which determines the ascorbic acid content of evaporated milk.

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A MODIFICATION OF THE LINE TEST APPLICABLE TO CHICKEN VITAMIN D ASSAY

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FOUR TEXT FIGURES AND TWO PLATES (SIX FIGURES)

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Remp and Marshall ('38), in a report on the antirachitic activity of various forms of vitamin D in the chick, made use of an "index of calcification" devised by one of us (E.H.) and obtained an excellent correlation between this index and the per cent of bone ash. They did not, however, describe the manner in which the index was determined. It is our purpose, in this paper, to describe the manner in which a rapid index of the degree of calcification in chick bones may be determined, by a modification of the U.S.P.XI line test, and to present data on the correlation of this index with the bone ash and the dosage of vitamin D. Although we have, in this laboratory, studied more than 700 chicks by the methods to be described below, the present report deals only with 240 birds which were put on test specifically for this paper.

OUTLINE OF TEST METHOD

The test animals were male white Leghorn chicks which were received in the laboratory in December, 1938, on the second day of life. They were separated into fourteen groups of twelve to eighteen birds each. The experimental procedures followed were those described by Remp and Marshall. The chicks were fed the modified diet of Hart, Kline and Keenan ('31), which was supplemented by 2% of corn oil for the negative control group, and 2% of cod liver oil (assaying

100 U.S.P. units of vitamin D per gram) for the positive control group. However, the chicks which received vitamin D supplements were medicated daily by direct (stomach tube) injection into the crop with the following exceptions: During the first 4 days (second to sixth day of life) none of the birds were given supplements in order to avoid excessive handling, but from the seventh to tenth days of life the daily vitamin dosage was doubled, thus compensating for the lack of initial treatment. After the tenth day of life the several groups of birds received the regular daily dosage at the levels indicated in the table. Body weights were recorded weekly, and the chicks were sacrificed on the thirtieth day of life. The two tibiae were removed, cleansed of soft tissues, and fixed in 90% ethyl alcohol for at least 48 hours, or until convenient for examination.

For our final selection of animals for inclusion in this report, we have used certain standards of growth. Only those birds have been included which gained at least 250% of their initial weight. We consider birds falling below this standard as too stunted to be representative. This criterion of selection leaves at least half the birds in each group, and in most of the groups leaves over 75%. From the selected chicks, one tibia was taken for the determination of bone ash as directed in the method of the Association of Official Agricultural Chemists (Lapp, '39), and the other tibia reserved for sectioning and staining as described below. The two assay procedures were carried out independently by the two authors, neither having knowledge of the results obtained by the other until the data were complete.

DESCRIPTION OF THE VITAMIN PREPARATIONS TESTED

The crystalline vitamin D_3 tested was prepared by the method of Windaus, Lettre and Schenck ('35). This compound has a formula of $C_{27}H_{44}O$, and a melting point of 82–84°C. The maximum absorption was at 265 mμ, the specific rotation in acetone was $[\alpha]^{20}_{D} = +83°$. The vitamin D_3-cholesterol corresponded in all respects to crystalline vitamin

D_3 with the exception that purification was less complete, so that the antirachitic potency was only 20,000 international units per milligram instead of 40,000 international units per milligram as in the case of the crystallized principle. The irradiated cholesterol in oil (Delsterol) was purchased on the open market in sufficient quantities of one lot for all tests. The antirachitic potency of all the preparations was determined initially by the standard U.S.P. rat method, using the U.S.P. Reference Oil and the International Standard for vitamin D.

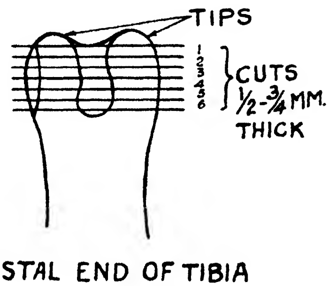

Fig. 1 Illustration of method of sectioning chicken bones

METHOD FOR THE DETERMINATION OF THE INDEX OF CALCIFICATION

The distal end of one of the paired tibiae from each chick is taken for the purpose of sectioning. The bone is cut with a razor blade, or other suitable instrument, at right angles to the long axis of the bone. After the condyles have been cut off—and a point is reached at which each cut includes an entire cross section of the bone—six or seven serial cuts of about 0.4 to 0.6 mm. in thickness are made, as illustrated by figure 1. This is as thin a cut as one can usually make by hand with a razor blade. The sections are immersed at once in 2% silver nitrate, and arranged serially; a shallow dish or watch glass may be used for this purpose. When the sections become suitably dark (time depending on the amount of light, but usually in 5 to 10 minutes), they are ready for evaluation. For this purpose a binocular microscope permitting 2 or 3

magnifications is desirable. Except for the method of making the cuts and the serial arrangement, the procedures differ in no important respect from the standard line test.

In order to evaluate the results, all of the sections are taken into consideration. If examination reveals that a questionable or borderline deviation from normal has occurred, the sections may be turned over and another reading taken. The principal points to be studied are: (1) size of the calcified areas, (2) density of calcification within these areas, whether solid or spotty (but in this connection one must not confuse unevenness of staining), and (3) shape of the calcified areas, whether well-formed or irregular in outline. A definitely normal bone, comparable to the positive controls, is recorded as 3+ (+++) to 4+ (++++). Calcification below this level is recorded as in the schema for the official line test. Negative controls show great variations in the degree of rickets present, but do not give anything comparable to plus readings. They are therefore recorded as (—), or (— —) if the rickets is very severe. A number of photographs illustrating several of the degrees of response are shown in the accompanying plates (figs. 2 to 7 inclusive). The amount of magnification used for each photograph was 2 times.

RESULTS

One tibia from each chick was used for visual examination as described above. The reliability of the modified line test as an index of the antirachitic effects of vitamin D was checked by determining the percentage of bone ash on the opposite tibia. These data, together with those on body weights, are presented in table 1.

Responses to the vitamin D_3 dietary supplement were the most typical. There was satisfactory growth at every dose level, and an increase in the average per cent bone ash for each increase in the vitamin supplement until the positive control level was reached. In contrast, the average index of calcification as noted visually continued to increase with each increment of vitamin supplement. The vitamin D_3-cholesterol

gave a satisfactory growth and the index of calcification likewise increased with each increase of vitamin supplement; but, the observed bone ash values are inconsistent since the average value at the 2-unit dose level exceeded those noted at either the 3-unit or 6-unit level. The reason for this is not clear. The irradiated cholesterol preparation gave a rather poor response in all particulars, especially in growth. Under the conditions of the experiment the positive control group also did not gain weight as rapidly as expected.

TABLE 1

Response of chicks to treatment with vitamin D

GROUP NO.	VITAMIN PREP'N.	DAILY DOSE	BODY WEIGHT Original median	BODY WEIGHT Gain, median	INDEX OF CALCIFICATION, AVERAGE	BONE ASH, AVERAGE
		I.U.[1]	*gm.*	%		%
1	Vitamin D_3	1	33	366	+1.38 (±0.77)	38.02 (±4.38)
2	,,	2	33	410	+2.56 (±0.33)	44.64 (±0.91)
3	,,	3	34	405	+3.05 (±0.18)	44.98 (±1.53)
4	,,	6	33	461	+3.38 (±0.30)	44.95 (±1.10)
5	D_3 cholesterol	1	32	433	+2.41 (±0.68)	43.40 (±2.02)
6	,,	2	32	435	+2.88 (±0.24)	45.61 (±1.24)
7	,,	3	32	398	+2.96 (±0.30)	44.03 (±0.86)
8	,,	6	34	469	+3.33 (±0.44)	45.11 (±0.87)
9	Irradiated cholesterol	1	34	294	+0.31 (±0.96)	36.29 (±2.68)
10	,,	2	33	354	+2.23 (±0.59)	42.02 (±2.35)
11	,,	3	34	378	+2.89 (±0.49)	44.03 (±2.05)
12	,,	6	36	298	+3.07 (±0.34)	45.64 (±1.02)
13	2% corn oil (diet alone)		36	300	−1.50 (±0.49)	31.57 (±0.76)
14	2% cod liver oil in diet		37	306	+3.34 (±0.31)	44.82 (±0.81)

[1] International units.

The chief purpose of this paper, however, is to emphasize the relationships which we have found to exist between the per cent of bone ash and our index of calcification, and of these factors in turn to the vitamin dosage. In order to bring out these relationships more clearly, they are presented in graphic form in figures 8, 9 and 10.

Figure 8 illustrates the observed relationship of dosage of vitamin D to the per cent of bone ash for all three prepara-

tions tested. The curves will be seen to be typical except in the case of the vitamin D_3-cholesterol, as mentioned above. It would be very difficult to decide from the bone ashes alone whether the prophylactic dose lies between 1 and 2 units or between 3 and 6 units. The irregularity noted might have been eliminated by using a larger number of birds per group than employed in this study, although we used the standard number (fifteen to eighteen).

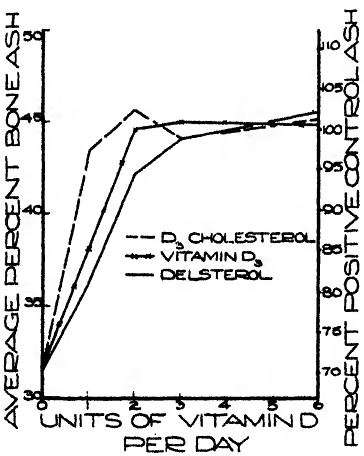

Fig. 8 Relationship of dosage of vitamin D to per cent of bone ash.

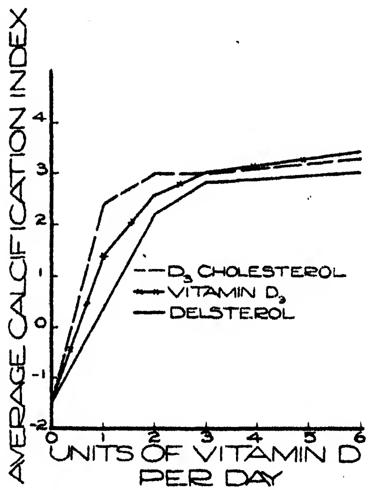

Fig. 9 Relationship of dosage of vitamin D to index of calcification.

Figure 9 illustrates the relationship of dosage of vitamin D to the index of calcification. The curves are much the same in character as those for the bone ash, showing an increase of the index with each increase of the vitamin supplement. One important difference between this factor and the observed ash values is worthy of mention: after a certain degree of calcification occurs, the bone ash may show only insignificant changes, while use of the suggested modification of the line test permits the better bone formation to be demonstrated.

Hence the index continues to rise after the observed bone ash has reached a plateau. We regard an index of calcification of +3 as normal and, with this as a basis, a clear-cut result may be deduced for the potency of each of the three preparations tested.

Figure 10 illustrates the relationship of the per cent of bone ash to the index of calcification as taken from the points given in the table. These points fall approximately on a straight line. However, in the case of individual chicks, there is not

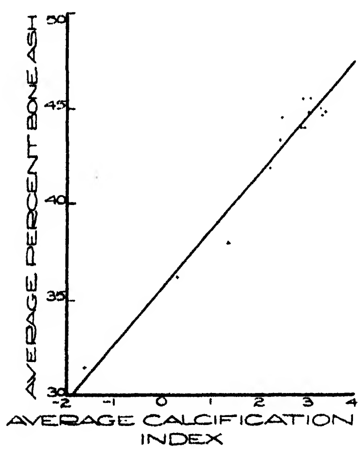

Fig. 10 Relationship of average per cent bone ash to average index of calcification.

always a good correlation between the two criteria, largely we believe because of greater variability in results obtainable by ashing methods. Thus a chick which gives a bone ash of 44% may give an index of +3.5, while we may find in another chick a bone ash of 47% and an index of +3.0. Nevertheless, the two criteria show in general a good correlation in the majority of cases and the mean values by the two methods approximate each other to an increasing degree with doses of 2 or more units daily.

DISCUSSION

Several criteria have thus far been used for vitamin D assay with chickens: (1) per cent of bone ash, (2) width of metaphysis, and (3) the index of calcification. Each of these has its value and all appear to give results which agree rather well with each other. We have not correlated the width of metaphysis with other indices in this report, but it was shown by Remp and Marshall to agree well with the bone ash and index of calcification. Of these three procedures, the bone ash method would ordinarily be the most time-consuming. It should be noted that the time required for the bone ash determination may be considerably reduced by ashing the bones in groups instead of individually, but even then the preliminary preparation involving cleaning, crushing, extracting and drying is a long procedure. The width of the metaphyses may of course be determined in less time than the index of calcification. Our experience indicates that this index will give information qualitatively as reliable as either of the other two criteria.

A further illustration of the extent to which the index of calcification and per cent bone ash are in agreement is seen in the conclusions which we would draw from each regarding the potency of the vitamin preparations tested in this report. If we consider an index of calcification of 3+ and a bone ash of 44.82% to be normal, as indicated by these assays, we arrive at the following conclusions as to the prophylactic doses of the three preparations: By bone ash, vitamin D_3, 2.5 I.U.; D_3-cholesterol, 1.7 (or 5.2) I.U.; irradiated cholesterol, 4.4 I.U. By the visual or line test index of calcification the corresponding doses are: vitamin D_3, 2.90 I.U.; D_3-cholesterol, 3.1 I.U.; Delsterol (irradiated cholesterol), 4.8 I.U.

SUMMARY

1. An adaptation of the U.S.P. line test to chick bones is described. The method permits a rapid and accurate determination of the index of calcification and an excellent corre-

lation between the degree of calcification and dietary supplements of vitamin D.

2. A typical chicken vitamin D assay is given including data on both the index of calcification and the per cent bone ash.

3. It is shown that both criteria would lead to substantially the same result for the potency of the various preparations of vitamin D tested.

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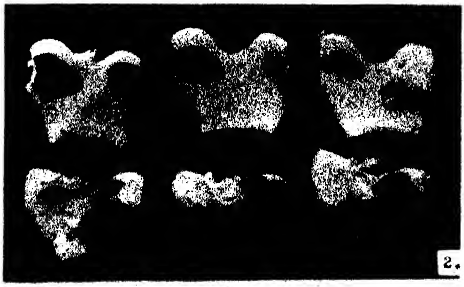
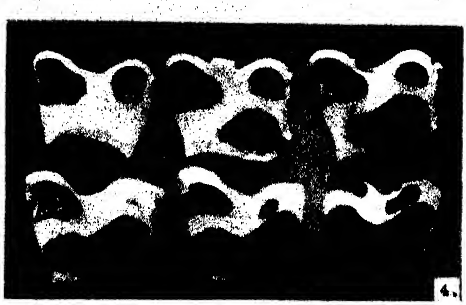
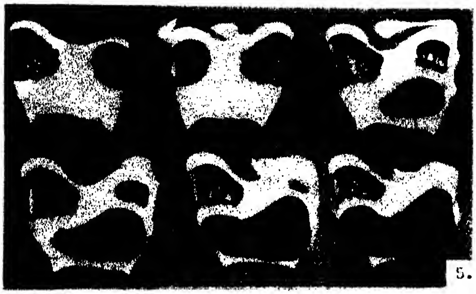




2 Index of calcification, —2; bone ash, 30.71%. Note almost complete absence of diaphyseal calcification in the first three sections, very poor and spotty diaphyseal calcification in the last three sections: poor bone formation.

3 Index of calcification, +0.75; bone ash, 36.67%. Note larger epiphyseal areas of calcification than above, but small diaphyseal areas except in the last section (and even this is not well-formed).

4 Index of calcification, +1.5; bone ash, 40.18%. Note the greater amount of diaphyseal calcification, first beginning to appear in the second section. There are large diaphyseal areas in the last three sections but none are well-formed.



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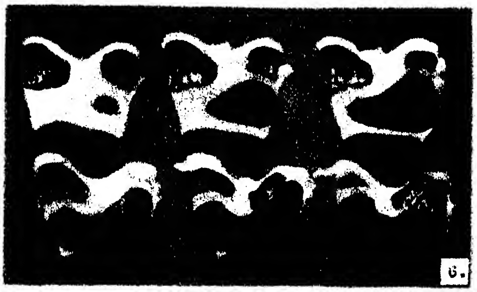

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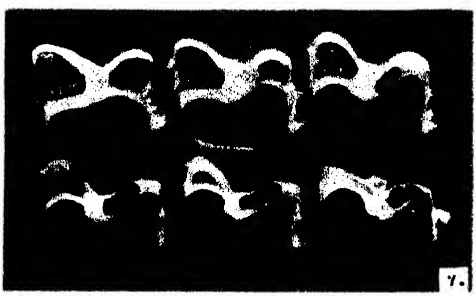

7.

5 Index of calcification, +2; bone ash, 42.80%. Note the diaphyseal areas of calcification do not begin to appear until the third section. However, those areas in the fifth and sixth sections are well-formed.

6 Index of calcification, +3.0; bone ash, 44.90%. Note that there are large diaphyseal areas of calcification in all but the first section. Excellent formation of bone is evident in the last three sections particularly.

7 Index of calcification, +3.5; bone ash, 47.80%. Note excellent bone formation throughout and the large well-formed diaphyseal area of calcification even in the first section. Bone was almost too hard to cut.

THE COMPOSITION OF GAINS MADE BY RATS ON DIETS PROMOTING DIFFERENT RATES OF GAIN [1]

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ONE FIGURE

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The rate of gain in body weight of the rat has been widely used in nutrition experiments as an important criterion of the adequacy of nutrition. During the last 20 years, as the increasing knowledge of dietary essentials has been applied to the feeding of laboratory animals, the rate of increase in body weight considered normal for this species has risen strikingly. Probably the most rapid rate produced by exclusively dietary means is that obtained by Anderson and Smith ('32). The present contribution includes an amplification of that work.

Another phase of development that has aroused interest is the ability of rats to grow after a period of stunting. According to Jackson and Stewart ('20), the nature, severity and duration of the deficiency as well as the age of the animal are factors affecting the rate of recovery from stunting. These generalities have been amply confirmed by Clarke and Smith ('38).

The purpose of the present investigation was to determine the composition, in terms of water, lipids, ash and protein, of the material added when rats gain in weight at different rates.

[1] The material for this paper was taken from a dissertation submitted by Marjorie Pickens in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Yale University, 1937.

The five rates represented were: the rapid rate obtained by Anderson and Smith ('32), that accepted as standard for this colony, two rates of retarded growth and an intermediate rate of gain obtained at a mature age during the recovery of the stunted animals.

EXPERIMENTAL

Male albino rats weighing from 38 to 46 gm. at 21 ± 1 days were divided into four experimental groups. For one group, referred to as the rapidly growing animals, the diet of modified calf meal,[2] paste food [3] and liberal vitamin supplements employed by Anderson and Smith ('32) was adopted in order to reproduce the rapid rate of gain observed by them. In addition to these foods, which were provided ad libitum, these animals received 3 gm. of dried yeast twice weekly and 20 gm. of lettuce daily.

For another group, representing a "normal" rate of gain, the stock diet of the colony was employed. Between the twenty-first and forty-second days this regimen was identical with that described above except that only 10 gm. of lettuce was given once weekly. From the forty-second to the forty-ninth day 3.5 gm. of paste food, approximately half the amount previously consumed, was allowed daily. On the forty-ninth day paste food was entirely withdrawn and calf meal remained the principal food. After the forty-second day the animals received 20 gm. of lettuce once weekly, 1 gm. of yeast three times weekly, and 1 gm. of wheat germ three times weekly.

A third group, gliadin-stunted rats, received ad libitum between the twenty-first and one hundred and tenth days a diet

[2] Maynard ('30). The modified calf meal used in this work was made up of 3% of cod liver oil and 97% of a mixture obtained from the Cooperative G. L. F. Exchange, Buffalo, New York, having the following percentage composition: Linseed oil meal 15, corn meal 20.75, ground malted barley 10, wheat red dog flour 22, dried skim milk 12, oat flour 15, soluble blood meal 3, salt 1, steamed bone meal 1, cod liver oil 0.25.

[3] The paste food consisted of 25% of whole milk powder, 25% of commercial casein, 20% of wheat germ and 30% of lard.

consisting of gliadin 18%, dextrin 51%, salt mixture (Osborne and Mendel, '19) 4%, hydrogenated vegetable oil [4] 22% and cod liver oil 5%. At the age of 110 days these animals were realimented by substituting an equivalent amount of lactalbumin for gliadin in the diet. The fourth group, calorie controls for the third, was fed between the forty-second and one hundred and tenth days this lactalbumin diet in the same amounts in which the gliadin-stunted rats voluntarily consumed their gliadin diet. After realimentation at 110 days both groups were fed the lactalbumin diet ad libitum. As a source of the vitamin B complex the stunted animals received throughout the experiment 200 mg. daily of dried yeast. Intended to be an adequate allowance at all ages, it was, in view of later assays [5] of this brand of yeast, probably inadequate in the latter period of the experiment.

Animals of all dietary groups were weighed and the weight of food consumed was noted every 4 days. Approximately eight animals were killed at birth and at 14 and 21 days of age, and eight were killed from each of the four dietary groups at 42, 110 and 230 days of age, and from the two stunted groups at 160 days as well.

The whole bodies freed of the contents of the gastrointestinal tracts were analyzed for water, fat, nitrogen and ash by modifications of methods employed by Light et al. ('34). The ground carcasses together with washings from the chopper were dried in vacuo at 55°C. to constant weight (0.1%). Lipids were determined on the entire ground carcass because it was found that accurate sampling for subsequent determinations was possible only on "dry fat-free matter." The material reported as lipids is that obtained by extracting the "dry matter" with boiling 4:1 mixture of anhydrous alcohol and ether, filtering, evaporating, dissolving the residue in ether, evaporating and drying overnight in a vacuum oven. All desiccations and evaporations were carried out under carbon dioxide and the solvents were freshly distilled in order to prevent oxida-

[4] Crisco.

[5] Private communication from Dr. G. R. Cowgill.

tion. The iodine number of the freshly isolated fat was determined by the Hanus method on a few samples of each diet and age group.

The "dry fat-free matter" was dried overnight in a vacuum oven, weighed and ground to a very fine, light powder in a ball mill or feed mill. Nitrogen was determined on 0.3 gm. samples of this material by the Kjeldahl method using metallic selenium as catalyst. Ash was determined on 1 gm. samples of the same material by incineration in a muffle furnace at 560°C.

RESULTS AND DISCUSSION

Increase in body weight. The growth curves of the four groups are given in figure 1. The rapidly growing animals gained at a slightly lower rate than those of Anderson and Smith ('32), requiring 25 days instead of 23.3 days to increase from 60 to 200 gm. in body weight. The records of the growth of the two stunted groups describe two levels of retarded rate of gain, and, in spite of differences in type of deficiency and in initial rate of growth, they show practically identical rates of recovery from stunting. Although the growth of recovery represents the most rapid growth of the stunted animals, it is distinctly inferior to the early gains of the rapidly growing and stock animals.

Recovery of food energy in the gains. Although this experiment was not designed to study the utilization of food energy, data on food intake were recorded and have been related to amounts of protein and lipids recovered in the gains. The energy content of the foods and of the protein and lipids of the gains was calculated, using the factors 4, 4 and 9 calories per gram for carbohydrate, protein and fat, respectively. For commercial products manufacturers' analyses were employed. As is to be expected, the proportion of food energy accounted for as fat and protein in the gains made by the two rapidly growing groups declined as these animals increased in weight. However, the stunted animals, as is indicated in table 1, showed some improvement in storage of food energy during

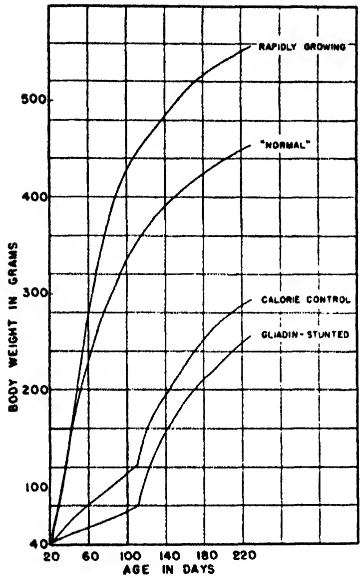

Fig. 1 Average growth curves of experimental groups.

TABLE 1

Recovery of food energy in gains made by stunted animals

EXPERIMENTAL GROUP	PERIOD (DAYS)	ENERGY CONSUMED IN FOODS		ENERGY ACCOUNTED FOR IN GAINS		PERCENTAGE OF FOOD ENERGY ACCOUNTED FOR IN GAINS AS LIPIDS AND PROTEIN
		Calories	Calories per gram of gain	As lipids (calories)	As protein (calories)	
Calorie controls	21– 42	412	18.7	...	18	2.1
	42–110	1169	20.5	54	47	8.6
	110–160	1945	19.0	148	70	11.2
	160–230	3438	50.5	114	56	4.9
Gliadin-stunted	21– 42	412	58.9	1	6	0.4
	42–110	1169	35.4	24	20	3.8
	110–160	1655	15.3	159	81	14.5
	160–230	3059	57.9	70	52	4.0

the period of deficient feeding. This response may imply an adaptation to the deficient diets.

Composition of bodies. The average values for the composition of the bodies of the experimental animals of the various groups are presented in table 2. The data for individual animals have been compared statistically by the method for small groups described by Snedecor ('37). The differences

TABLE 2

Average composition of bodies

EXPERIMENTAL GROUP	AGE	NET WEIGHT	COMPOSITION OF WHOLE BODIES					COMPOSITION OF FAT-FREE MATERIAL		
			Water	Lipids	Protein (N × 6.25)	Ash	Dry fat-free matter	Water	Protein	Ash
	days	*grams*	%	%	%	%	%	%	%	%
	Birth	6.23	86.06	1.78	9.01	1.44	11.72	87.6	9.2	1.5
	14	22.30	73.61	9.36	13.24	2.20	16.79	81.2	14.6	2.4
	21	39.9	67.98	13.20	13.69	2.08	17.42	78.3	15.8	2.4
Rapidly growing	42	161.9	64.4	13.8	16.6	2.2	20.97	74.7	19.3	2.6
	110	445.9	57.0	21.1	16.2	2.6	20.55	72.2	20.5	3.2
	230	540.9	53.4	24.7	15.8	2.7	19.93	70.9	21.5	3.6
Stock	42	147.1	65.9	12.5	16.5	2.5	21.29	75.3	18.9	2.9
	110	350.3	62.0	13.9	19.3	3.2	24.02	72.0	22.4	3.7
	230	439.2	57.5	17.2	17.7	3.0	22.87	69.4	21.4	3.6
Calorie controls	42	62.1	69.1	6.8	16.2	3.2	22.13	66.6	17.4	3.4
	110	119.0	66.7	8.6	18.3	4.0	24.14	73.0	20.0	4.4
	160	221.3	63.6	12.0	17.7	3.5	23.18	72.3	20.1	4.0
	230	289.3	61.3	13.6	18.4	3.6	23.83	71.0	21.3	4.2
Gliadin-stunted	42	46.5	66.3	11.6	15.1	3.4	20.88	75.0	17.1	3.9
	110	79.1	65.9	11.5	14.9	3.9	20.50	73.4	16.7	4.3
	160	187.1	62.4	13.8	17.2	3.5	22.30	72.4	20.0	4.1
	230	249.0	61.6	13.5	18.1	3.5	23.46	71.2	20.9	4.1

mentioned in the following discussion were found to be significant according to this treatment.

The rapidly growing and stock animals, fed similarly to the age of 42 days, showed no significant differences in composition at that age. At 110 days the former had a markedly greater fat content; at 230 days this difference though still present was somewhat reduced, owing chiefly to the increase in fat content of the stock rats.

The gliadin-stunted group contained at 42 days a proportion of fat comparable to that of the rapidly growing and stock rats. The calorie controls, as might be expected, contained little fat but proportionately more protein, resembling the two heavier groups in protein content. At 110 days both stunted groups differed strikingly from the rapidly growing animals in containing more water and less fat. No such replacement of fat by water was observed in gliadin-fed albino mice by Mendel and Judson ('15). Mice restricted in caloric intake, however, were found by these investigators to contain the same proportion of fat as younger normal mice of the same weight. Values relatively high for water and low for fat have been observed in animals stunted by other types of deficiencies (Mendel and Judson, '15; Light et al., '34; Johnson et al., '36). After realimentation the two stunted groups of this experiment approached the same composition but were still poorer in fat than the heavier groups.

Several investigators have commented upon various ratios and values for certain fractions in animal bodies. Moulton ('23), Armsby and Moulton ('25) and Johnson et al. ('36) have adduced evidence for the comparative constancy of the ratio of water to protein in animal bodies after chemical maturity (Moulton, '23) has been reached. Johnson and co-workers have calculated these values from the data furnished by other workers and have found that for mature rats reported averages vary from 2.89 to 3.95. In the present study the values for this ratio, which are derived from data shown in table 2, are indeed relatively constant, varying between 3.2 and 3.5 for "normally" and rapidly growing animals after chemical maturity (50 days of age) has been reached. The stunted animals during the period of inadequate nutrition showed high values for this ratio, a variation in the direction characteristic of younger animals. After realimentation this ratio changed in the direction of a normal value, but still remained slightly above that of the heavier groups.

The proportion of dry fat-free ash-free material in the bodies has been computed by Chanutin ('30) and by Light et al. ('34), the former reporting an average value of approxi-

mately 20%; the latter, 19.8%. Values derived from table 2 vary from 16.6% to 20.9% for mature animals of all dietary groups. The low figure refers to the gliadin-stunted group of 110 days; it varies in the same direction as the still lower figures for suckling young.

Armsby and Moulton ('25) calculated that the dry fat-free ash-free material contains approximately 16.05% of nitrogen. Chanutin ('30) and Light et al. ('34) reported this value as 15.9 and 15.3 respectively. Still lower figures found in the present investigation may be calculated from table 2. Averages for mature animals of all dietary groups vary from 14.3% to 14.8%; those for young animals on inadequate diets are slightly below 14.0%.

The variability of the fat content has led a number of investigators to express the composition of bodies on a fat-free basis. Murray ('19, '22) stated that the fat-free matter is of practically constant composition. Moulton ('23), by calculating values for the composition of bodies to the fat-free basis, was able to define regular and consistent changes with age. The group averages for the body composition of animals of this experiment, calculated to the fat-free basis, are included in table 2. Comparison with values for whole bodies makes clear the greater uniformity of composition expressed on this basis; most striking is the very close similarity of all four groups at 230 days.

Iodine number of body lipids. The iodine numbers of the body lipids are recorded in table 3. Obviously the most saturated fat was obtained from rats at birth. No significant differences appear in the average values for age groups of animals on different dietary regimens. These data do not illustrate the "hardening" of body fat with increasing weight (advancing age) observed by Anderson and Mendel ('28) in rats on a diet composed of two-thirds whole wheat and one-third dried whole milk. They suggest that rate of gain in body weight has little if any influence on the degree of unsaturation of the body lipids.

Composition of gains. Table 4 presents the percentage composition of the gains calculated from the group averages

TABLE 3

Iodine number of body lipids

EXPERIMENTAL GROUP	AGE (DAYS)	IODINE NUMBER (AVERAGE FOR GROUP)
	Birth	51.7
	14	64.1
	21	64.8
Rapidly growing	42	62.8
	110	67.5
	230	69.5
Stock	42	66.3
	230	69.3
Calorie controls	42	67.1
	110	69.6
	160	67.0
	230	68.5
Gliadin-stunted	42	70.1
	110	73.5
	160	65.7
	230	68.0

TABLE 4

Composition of gains

EXPERIMENTAL GROUP	PERIOD	GAIN IN NET WEIGHT	WATER	LIPIDS	PROTEIN	ASH
	Days	*gm.*	%	%	%	%
	0– 14	16.07	68.8	12.3	14.9	2.5
	14– 21	17.6	60.8	18.1	14.3	1.9
Rapidly growing	21– 42	122.0	63.2	14.0	17.5	2.2
	42–110	284.0	52.8	25.3	16.0	2.8
	110–230	95.0	36.5	41.6	13.9	3.2
Stock	21– 42	107.2	65.1	12.2	17.5	2.7
	42–110	203.2	59.2	14.9	21.3	3.7
	110–230	88.9	39.8	30.2	11.4	2.2
Calorie controls	21– 42	22.2	71.1	..[1]	20.7	5.3
	42–110	56.9	64.1	10.6	20.6	4.9
	110–160	102.3	60.0	16.0	17.0	2.9
	160–230	68.0	53.8	18.8	20.7	3.9
Gliadin-stunted	21– 42	6.6	56.2	1.8	23.6	11.4
	42–110	32.6	65.3	11.4	14.6	4.6
	110–160	108.0	59.8	15.5	18.9	3.5
	160–230	61.9	59.2	12.6	20.8	3.5

[1] The average lipid content in grams decreased during this interval.

for net weight and body composition. Mitchell and Carman ('26) have shown by computing the gains of individual rats that equal gains made at the same rate by litter-mates on the same diet may differ widely in composition. In the present experiment individual variation has been taken into account by the statistical treatment.

The trends found in the composition of bodies are magnified in the composition of gains. With a few exceptions, the proportion of fat increased and that of water declined with advancing age. No striking relationship is noted between age and proportion of protein or of ash in the gains.

Considered in terms of groups, these data on the gains show the tendency of the rapidly growing animals to accumulate greater proportions of fat and smaller proportions of water than the animals of any other groups. A tendency to higher values in protein content is found in the stunted animals. Among figures for ash content those for the stunted groups, especially during the period of inadequate feeding, are definitely high.

The results of this experiment relating to the fat content of the gains are in accord with those of Moulton, Trowbridge and Haigh ('22), which have been enhanced by Mitchell's ('29) mathematical treatment of the data. Steers were maintained at three planes of nutrition by different amounts of the same feed. It was found that the best nourished and most rapidly growing group added the greatest proportions of fat at all ages and the most slowly growing group contained and deposited the least fat. A similar statement holds for the two heavier groups of the present experiment. Contrary evidence was noted by Mitchell and Hamilton ('29) in hogs fed ad libitum a uniform diet. Individual variations in rate of gain were not paralleled by consistent differences in body composition.

Haecker ('20), who analyzed the bodies of steers over a wide range of weights, distinguished between two periods, the "growing stage," when protein exceeded fat in the gains, and the "fattening stage," when the reverse held. Similarly

Moulton and co-workers ('22) observed a "thinning" during early growth followed by a tendency to deposit fat after the period of most rapid gain in weight. They found that nitrogen storage followed that of water and was inversely proportional to fat deposition. With respect to the two adequately fed groups, the present experiment agrees with those of Haecker and of Moulton et al. Early rapid gains contained larger proportions of protein and smaller proportions of fat than later slower gains.

The stunted animals of the present experiment, however, experienced early slow growth and later rapid growth. The slow gains contained a preponderance of protein over fat, which was particularly striking during the first period studied. During realimentation, the period of most rapid growth of the stunted animals, the greatest percentage of fat appeared in their gains. Rate of gain in weight does not necessarily run parallel to preponderance of protein over fat in the gains.

One factor of importance is age; some of the earliest works on the composition of bodies brought out the influence of age on fatness. According to Reed, Yamaguchi, Anderson and Mendel ('30), fatty acid deposition is more closely correlated with body weight than with age. In the present experiment older stunted animals, however, made gains containing greater proportions of fat than did younger "normal" animals of the same body weight. The data might be interpreted to mean that in the early stages of "normal" growth the emphasis is placed upon increase of active body tissue, represented by protein, at the expense of fat deposition, whereas at mature ages the emphasis is on the accumulation of fat.

Another important influence is diet. This factor was not controlled for the rapidly and "normally" growing groups because it was desired to reproduce conditions studied previously. Whereas the caloric intakes of these two groups were similar, their diets differed in composition. The rapidly growing animals selected a mixture containing about 35% of protein and 35% of fat, while the "normal" group received about 18% of protein and 7% of fat. This difference in protein

levels may not have exerted a significant influence on the composition of the gains. Hamilton ('39) observed in controlled feeding experiments that differences in amount of whole egg protein ranging from 16% to 42% of the diet failed to cause marked differences in the composition of the gains. On the other hand, the difference in the amounts of lipids ingested undoubtedly affected the results of the present experiment. This is indicated by the work of Reed et al. ('30) and of Paquin ('35) which shows that dietary fat promotes fat deposition in rats to a greater extent than does carbohydrate. Since rate of gain is related to diet and since diet influences body composition, the relation between rate of gain and body composition is inevitably complicated.

The possibility of mild vitamin B_1 deficiency in the two stunted groups during the latter part of the period of realimentation cannot be overlooked. The work of McClure, Voris and Forbes ('34) has established the role of vitamin B_1 in promoting fat deposition. Even a degree of deficiency too mild to be detected by the growth curve was shown to interfere with the storing of fat. The tendency of the stunted animals to deposit more fat than protein during the period of their most rapid growth has probably not had full expression in this experiment. More striking results might have been obtained if an abundance of vitamin B_1 had been assured as the animals increased in size.

Early students suggested that the function of growth is retained only by exercising it, a conception soon disproved. In the present experiment those animals that achieved the most rapid early growth, in which water and protein were added in high proportions, accumulated in maturity disproportionate quantities of fat in the gains. In contrast, the stunted animals, given an opportunity for growth only at a mature age, made gains richer in protein and water than those made at the same age by the heavier animals. If previous performance has any effect on later growth, it appears that early suppression of growth may cause subsequent persistent

gains in lean tissue at the expense of some of the fat that is added in large amounts by normal animals of mature age.

SUMMARY AND CONCLUSIONS

Rates of gain including comparatively rapid early gain of rapidly growing and "normal" rats, the slow later increase of the same animals, the retarded early growth of two stunted groups and a more rapid increase in weight made by the stunted animals at a mature age, were produced by dietary adjustment.

Results relating to the rapidly growing and "normal" groups show that during early growth gains were rapid and contained a preponderance of protein over fat, while later gains were made considerably more slowly and were accompanied by the accumulation of large quantities of fat. The performance of the stunted animals, on the other hand, demonstrates conclusively that ratio of protein to fat in the gains does not, as has been suggested, run parallel to the rate of increase of body weight. During early life, when gains were slight, the stunted animals added much more protein than fat. After realimentation, when their most rapid increase in weight took place at a rate greater than that of the heavier groups of the same age, these animals added considerably more fat than previously. No single generality, therefore, relates rate of growth and composition of gains, but other factors such as age and nature of the diet appear to influence the composition of the gains.

The composition of the whole bodies of the four groups reflected the trends described for gains. The fat-free matter of the bodies showed smaller variations. At the end of the experiment, although the whole bodies differed considerably in size and composition, the fat-free material of the four groups was of practically identical composition.

The iodine number of the body fat rose sharply between the first and fourteenth days. Thereafter this value did not differ significantly for animals of different age and dietary history.

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THE INFLUENCE OF FAT ON CALCIUM AND PHOSPHORUS METABOLISM

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Numerous reports have appeared during the past several years dealing with the influence of fat on the metabolism of calcium and phosphorus. Holt, Courtney and Fales ('20) found that the utilization of calcium by infants was augmented by fat in the diet. Hickmans ('24) has reported similar results. An increased absorption of calcium and phosphorus from the intestines of rats was observed by Boyd, Crum and Lyman ('32) when fat was added to the diet. Several investigators have studied the influence of fat on the development of rickets in rats. Zucker and Barnett ('23) have reported that cottonseed oil and its hydrogenated product[1] showed antirachitic properties although it was impossible to concentrate the antirachitic activity in the non-saponifiable fraction. From a series of investigations on butterfat, lard, egg oil and olive oil, and the fatty acids prepared from butter and lard, Kon and Booth ('33, '34 a, '34 b) have concluded that the antirachitic activity of the saponifiable fraction of a fat is due to the fatty acids and not to a specific compound. McDougall ('38) employing a diet deficient in calcium found that rats were protected against the low-calcium type of rickets by including 11% lard in the diet. Olive oil and cocoanut oil were less effective. Palmer and Mottram ('39) were unable to confirm the findings of McDougall. Gridgeman, Lees and

[1] Crisco.

Wilkinson ('39) observed that fat in the diet influenced the degree of new calcification produced by vitamin D.

In the following experiments the relation of fats to calcium and phosphorus metabolism has been further studied. Increased calcification in the long bones of rats on an otherwise rachitogenic diet has been used as a measure of the beneficial effect of the fats, and several of the possible modes of action suggested by the above-mentioned investigators have been tested. The investigations were facilitated by the use of synthetic rachitogenic diets in which it is possible to vary certain constituents of the diet without disturbing the quantitative relationships of the others. This is of particular importance in the study of rickets in the rat inasmuch as the ratio as well as the absolute amounts of calcium and phosphorus plays a significant role.

EXPERIMENTAL

Albino rats at approximately 25 days of age were placed on synthetic rachitogenic diets similar to those previously described (Jones, '39 a). The fat, or related substance, to be studied for its calcifying properties was added to the ration at the expense of the carbohydrate. In some experiments the substitution was made on a weight basis and in others on a calorie basis. With only a few exceptions, the basal diet without substitution was given to a group of rats as controls simultaneously with the feeding of the experimental rations to the test animals. The usual precautions were taken to distribute the animals with respect to sex and litter mates among the various groups. After about 21 days on the experiment the rats were bled and the pooled sera (usually from three animals) were analyzed for calcium by the method of Clark and Collip ('25) and for inorganic phosphorus on the calcium-free filtrate by the method of Gunther and Greenberg ('29). The right femurs were removed and the percentage of ash determined on the dry, lipoid-free bone. The wrists were removed for examination by the "line test" technic.

In table 1 are summarized the results of feeding various quantities of lard as a supplement to a rachitogenic diet. The first seven groups of animals were given diet no. 570 (Jones, '39 a) modified to the extent of reducing salt no. 5 to 5%.

TABLE 1

The effect of lard on bone ash and serum calcium and phosphorus of rats on rachitogenic diets

GROUP NO.	SALT NO.	LARD	NUMBER OF ANIMALS	FEMUR ASH		BLOOD SERUM		
						Number of determinations	Ca	P
Yeast								
		%		mg.	%		mg. per 100 cc.	mg. per 100 cc.
1	5	0	23	20.0	25.0	8	12.0	2.9
2	5	5-W[1]	9	30.6	31.1	3	14.1	3.6
3	5	10-W	6	35.1	33.9	2	13.6	4.6
4	5	25-W	15	34.7	34.3	5	15.1	4.6
5	5	10-C[1]	6	32.8	34.1	2	14.0	4.7
6	5	25-C[1]	3	38.0	37.5	1	14.0	7.0
7	5	50[2]	3	20.8	23.1	1	12.0	2.0
8	10	0	13	21.9	26.0	4	11.3	3.8
9	10	10-W	3	34.7	33.6	1	13.3	5.3
10	10	25-W	3	39.2	35.2	1	13.7	6.1
Liver extract + thiamin								
11	5	0	12	16.5	21.3	4	12.6	2.3
12	5	5-W	9	19.8	22.5	3	12.9	2.8
13	5	10-W	9	21.8	25.5	3	13.7	3.1
14	5	25-W	15	21.2	27.0	4	15.6	3.5
15	10	0	12	17.7	20.5	4	11.7	2.5
16	10	5-W	9	18.4	20.4	3	12.3	3.0
17	10	10-W	7	21.4	26.4	2	13.2	3.9
18	10	25-W	12	17.4	23.4	4	13.6	4.6

[1] See text for explanation.

[2] The non-saponifiable extract of the equivalent of 50% lard.

Diet no. 570 is composed of the following ingredients expressed in per cent: alcohol extracted fibrin 18; yeast 5; salt no. 5, 5.3; agar 2.0; carotene solution 0.1 and dextrin 69.6. In some of the experiments glucose[2] was used in place of the dextrin. This diet contains a little over 1% calcium and 0.09% phos-

[2] Cerelose.

phorus. The data obtained on the control animals (those receiving no lard) are summarized as averages and designated as group 1. The other groups received lard as indicated. The letter W following the amount of fat given denotes that an equal weight of carbohydrate was replaced by lard. The letter C indicates that an adjustment of the other constituents of the diet was made to allow for the increase in calories in the lard; that is, the lard still furnished a given portion of the diet by weight but all other constituents (except carbohydrate) were so increased at the expense of the carbohydrate as to keep unchanged the amounts of these constituents in unit calorie portions of the diet. The data show clearly that even as little as 5% of lard in the diet definitely increased calcification under these conditions. In general, in the animals receiving the lard there was an increase in both the absolute and relative amounts of ash in the femurs, and an increase in both the calcium and phosphorus of the serum. Examination of the distal end of the radii by the "line-test" technic also showed increased calcification. The bones were still not normal as revealed by this examination and could still be classed as rachitic, but the uncalcified areas were much narrower in the fat-fed animals than in the controls. Also the margins of these areas were less regular and the surrounding calcified portions appeared much denser. The results were the same whether the lard was given on a weight or calorie basis.

Group 7 was given, in addition to the basal diet, the unsaponifiable fraction of 50 gm. of lard per 100 gm. of diet, or an equivalent of 50% of the original fat. None of the criteria used in these studies indicated any increase in calcification as a result of this addition. This shows that the increased calcification produced by the lard was not due to the presence of vitamin D. Furthermore, as the lard contained only traces of phosphorus (approximately 0.002%) the results cannot be due to an increase of this element. The next three groups of animals (groups 8, 9 and 10) were given a basal diet containing 3% of salt no. 10 (Jones, '39 b). This salt mixture differs from salt no. 5 only in respect to its content

of calcium carbonate and when fed at a 3% level furnished about 0.38% calcium while the other ingredients of the salt mixture remained the same. Here again 10 and 25% of lard (the only two levels studied) definitely increased calcification.

In groups 11 to 18 inclusive, experiments very similar to those described above were repeated except that the vitamin B factors were supplied by liver extract [3] (usually at a 2% level) and 200 µg. of thiamin hydrochloride [4] per 100 gm. of diet. This change reduced the amount of phosphorus in the diet from about 0.09% to slightly more than 0.02%. As can be seen, the influence of fat on calcification was less marked when the phosphorus deficiency was made more severe. The serum calcium and phosphorus seem to have been affected by the lard to a greater extent than the bone ash especially when the fat was given at the 10 and 25% levels. Examination of the radii by the "line test" technic also indicated greater calcification than would have been predicted from the bone ash. This is in line with the recent report of Bunker, Harris and Mosher ('40) who found that the "line-test" and x-ray are more sensitive indicators of increased calcification than is the percentage of bone ash. Although no data are presented it was found that cottonseed oil and hydrogenated cottonseed oil [5] also definitely increased calcification. These two fats were fed at only the 25% level. Uncombined oleic acid [6] was about as effective as lard. On the other hand, the animals failed rapidly when 25% of stearic acid was included in the diet. Considerable amounts of this acid were excreted in the feces, partially in the form of the calcium soap. The loss of calcium was sufficient to produce a hypocalcemia with a concomitant rise in serum phosphorus. Several of these animals died within 3 weeks after being placed on the diet. Neither sodium oleate nor the calcium soaps of lard had any

[3] The author is grateful to Dr. C. E. Graham of The Wilson Laboratories, Chicago, Illinois, for supplying the alcohol-extracted fibrin and the liver extract used in these experiments.

[4] Kindly furnished by Merck and Co., Inc., Rahway, N. J.

[5] Crisco.

[6] U. S. P. Merck.

apparent effect on calcification under these conditions. In table 2 are summarized the results of an experiment in which seventeen rats were divided into five groups. One group was given the basal diet containing 5% of yeast and 5% of salt no. 5. The other groups were given supplements as indicated in the table. All supplements were fed at a 10% level with adjustments of other constituents on a calorie basis as described above. In the diet containing the calcium soaps of lard the calcium carbonate of the salt mixture was decreased to an extent equivalent to the amount of the calcium added as the soaps, thus preventing any change with respect to this element. As judged from bone ash the lard and oleic acid

TABLE 2

A comparison of the effects of lard, oleic acid, sodium oleate and calcium soaps of lard on bone ash and serum calcium and phosphorus

SUPPLEMENTS TO BASAL DIET	NUMBER OF ANIMALS	BONE ASH		BLOOD SERUM	
				Ca	P
		mg.	%	*mg. per 100 cc.*	*mg. per 100 cc.*
None	3	18.3	24.6	12.2	2.9
Lard	3	33.8	36.2	15.0	5.8
Oleic acid	3	26.4	31.2	15.0	3.1
Na oleate	4	19.6	25.3	11.4	3.3
Ca soaps	4	17.9	24.3	14.1	3.3

definitely increased calcification, whereas sodium oleate and the calcium soaps of lard were without effect. There is some discrepancy in the calcium and phosphorus values of the serum. It appears that the oleic acid failed to increase the level of phosphorus whereas the calcium soaps increased serum calcium. The compositions of the sera of these two groups are, therefore, almost identical, although there was greater calcification in the former than in the latter case. However, no significance can be attached to single determinations as there is considerable individual variation in the concentration of these elements and the values are dependent on several factors. The averages of two other determinations on sera from animals receiving oleic acid were: Ca, 13.0 and P, 4.2 mg.

per 100 cc. Increased calcification in the animals receiving the lard and oleic acids as compared to the other groups in the experiment was also indicated by examination of the wrist bones according to the "line-test" technic. Several additional experiments have confirmed the above observation that under the prevailing conditions, oleic acid definitely shows antirachitic properties whereas sodium oleate and the calcium salts of lard do not.

DISCUSSION

In the light of the data presented above it is now possible to reconsider some of the theories advanced as possible explanations for the manner in which fat increases the utilization of calcium and phosphorus. Zucker and Barnett ('23) believe that a fat acts as an antirachitic by uniting with the calcium to form soaps of the fatty acids and thus liberating a larger portion of the phosphorus. If that were the case some antirachitic effect should have been obtained by simply decreasing the amount of calcium in the diet. Direct comparisons were made between salt no. 5 at a 5% level (1.1% calcium) fed with 10% lard and salt no. 10 at a 3% level (0.38% calcium) fed with no lard. Assuming the average molecular weight of the fatty acids of lard to be equal to that of oleic acid and that all of the acids united with calcium, 10% of lard would combine with only 0.7% of calcium. There is thus about 0.4% of calcium still available to combine with the phosphorus. This is equal to the amount in the diet containing salt no. 10 and no lard. Without exception the greater calcification took place on the diet containing the lard. On the other hand, when the calcium soaps of lard were fed and the calcium of the basal diet was adjusted to allow for the added calcium, rickets was produced. These results seem to disprove the theory of Zucker and Barnett. According to McDougall's ('38) idea, fats act by forming the calcium soaps and by so doing increase the absorption of this element. The experiment with the calcium soaps just referred to apparently contradicts this theory.

According to the opinion expressed by Gridgeman, Lees and Wilkinson ('39) fats act indirectly by lowering the consumption of salts. The experiments of these authors are open to criticism as a constant intake of calcium and phosphorus was not maintained when the fat was added to the diet. It is in experiments of this kind that a basal diet composed of individually purified materials can be employed to considerable advantage. As described above, experiments were performed in which the lard was substituted for the relatively pure carbohydrate on both a weight and calorie basis. In both types of experiments the ratio of calcium to phosphorus remained unchanged, and in the latter the amounts consumed varied only as the intake of calories varied. Although daily food consumption was not measured there was no obvious increase or decrease in the amount of food eaten when fat was added to the diet. In a few cases the rats getting the fat gained slightly more weight than their controls, but usually there was no significant difference in this respect. It is, consequently, improbable that in these experiments the antirachitic action of the lard was the result of an altered intake of either calcium or phosphorus.

Boyd, Crum and Lyman ('32) explain the action of fat on calcification on the basis that it increases the acidity of the intestinal tract with subsequent greater absorption of calcium and phosphorus. The fact that lard and oleic acid increase calcification while sodium oleate and the calcium salts of lard do not is suggestive evidence in favor of this view. The results of experiments in progress at the present time, in which the effect of various calcifying agents on the hydrogen ion concentration of the intestinal tract is being studied, will be reported later.

Regardless of the mode of action, the above data show clearly that, aside from their content of vitamin D, fats exert a definite calcifying action in the rat. The response of the rat to the various factors influencing calcium and phosphorus utilization is somewhat different from that of most mammals. Whether this difference holds for fats remains to be deter-

mined. If there should be a variation in this respect the antirachitic value of high fat-low vitamin D foods as determined on the rat may not be entirely applicable to other animals. Lard, to the extent of only 5% of the diet, showed definite antirachitic properties especially when given in conjunction with the basal diet containing the higher amount of phosphorus. Whole corn contains nearly this much fat. It is possible that the occasional difficulties encountered in the production of rickets on the Steenbock-Black ('25) diet may be due in part to an exceptionally large amount of fat in the particular sample of corn being used.

SUMMARY

Lard when added to synthetic rachitogenic diets in amounts varying from 5 to 25% had definite antirachitic properties which were not associated with the non-saponifiable fraction. The lard increased calcification on diets containing excessive (1.1%) or moderate (0.38%) amounts of calcium. The lard was effective when added on either a weight or calorie basis. The effect of the lard was less pronounced on diets very low in phosphorus (0.02%) than on diets higher in phosphorus (0.09%). Oleic acid also increased calcification but sodium oleate and the calcium soaps of lard showed no antirachitic action. These data are discussed in relation to the various theories advanced to explain the action of fats in favorably influencing calcium and phosphorus utilization. With the exception of the effect on the acidity of the intestinal tract these theories are apparently disproved by the data here reported.

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THE INFLUENCE OF DIETARY RIBOFLAVIN ON THE CONTENT OF THIS VITAMIN IN CHICKEN TISSUE

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Several reports have been made on the riboflavin or vitamin G content of meats. Many of the earlier attempts to quantitatively measure this vitamin in meat and meat products were measurements of a part of the vitamin B complex rather than a specific measurement of riboflavin. The isolation and synthesis of riboflavin made possible new and better methods for the determination of riboflavin. In 1938 Darby and Day reported the riboflavin content of meat as determined by the rat growth method. In 1939 Mickelsen, Waisman and Elvehjem made a more extensive study of the same problem by use of a microbiological method of measurement. The results of these two studies are in good agreement and are comprehensive enough to give a fairly complete picture of the riboflavin content of the meats which are used for human food. However, these reports have not included any attempt to study the factors affecting the riboflavin content of meat. The development of the microbiological method of Snell and Strong ('39) and the fluorometric method of Hodson and Norris ('39) for determining riboflavin have made possible studies of this character, since these methods are rapid, accurate, and require only a small amount of material as a sample. The microbiological method has been used by Fraser, Topping and Isbell ('40) to study variations in the riboflavin content of tissues of rats and dogs fed diets deficient and adequate in this vitamin. Since neither of these species is of economic importance as a source of meat, a further investigation has been undertaken.

The chicken has been used for this investigation since riboflavin is very important for this species as pointed out by Norris and others ('36) and by Schumacher and Heuser ('39), and because the chicken is small in size it can be kept under controlled dietary and environmental conditions at a small cost. While the results obtained in such an experiment are primarily applicable only to the one species, they also serve as pilot observations suggesting work with other animals.

EXPERIMENTAL

Groups of Single Comb White Leghorn chicks were placed both on a diet deficient in riboflavin and on one containing an excess of this vitamin, and the riboflavin content of the tissues and organs was determined. The percentage composition of the basal diet was as follows: table corn meal 55.5, peanut oil meal 15.0, casein 11.0, cane molasses 5.0, salt mixture 5.0, soybean oil 3.0, fortified cod liver oil 0.5, and wheat bran 5.0. The diet for the group of chicks receiving an excess of riboflavin was composed of the basal ration 95% and yeast 5%. To each gram of feed 4.34 µg. of synthetic riboflavin were added. Analyses indicated that the diet contained 6.9 µg. of riboflavin per gram. The diet deficient in riboflavin was composed of 99% basal diet and 1% of yeast. Analyses indicated that this ration contained 1.0 µg. of riboflavin per gram.

The chicks were maintained on the diets for 6 weeks and were then killed and the tissues removed. The samples were weighed, dried in hot air at 50 to 60°C. for 24 hours, reweighed and ground. The riboflavin content of the tissues and feed was determined by the fluorometric method of Hodson and Norris ('39). Samples of liver, heart, gizzard, leg, and breast from the two sources were compared. In addition riboflavin determinations were made on two samples of spleen, two samples of eye, and one sample each of lung, comb and brain from the chickens receiving the excess of riboflavin. The entire liver, gizzard, heart, eye, spleen, brain, comb or lung was used for a sample. In a few cases organs from two or more birds were combined to form a sample. The hearts from chickens on the

low riboflavin diet were very small and were, therefore, combined to form a single sample. The breast samples were from a single bird. The leg samples consisted of all the muscular tissue surrounding the femur and tibia on one leg of a bird and constituted the cuts usually designated as the thigh and drumstick. The breast samples represented light meat and the leg samples dark meat.

The results which are presented in table 1 indicate that the amount of riboflavin in the diet of chickens has a marked influence on the riboflavin content of their tissues and organs. The tissues and organs of the birds fed a diet containing an excess of riboflavin contained from two to four times as much riboflavin as those from birds fed a ration deficient in this factor. The riboflavin content of a single tissue from chickens on the same diet also shows some variation (table 1). This individual variation is the most marked in the case of the leg tissue. However, the riboflavin content of the leg samples from the chickens on the excess riboflavin diet is as high or higher than that reported for other muscular meats by Mickelsen, Waisman and Elvehjem ('39). In agreement with their data the results presented in table 1 indicate that the white breast meat has a very low riboflavin content. Riboflavin determinations on tissue from five birds fed a commercial diet indicated that the leg tissue and breast tissue contained respectively 9.3 μg. and 3.6 μg. per gram of dry tissue. These results are also in excellent agreement with those reported by Mickelsen, Waisman and Elvehjem for chicken meat from birds on a normal ration.

The results of determinations on two samples of spleen, two samples of eye, and one sample each of lung, comb, and brain, indicated that these tissues contained 23.2, 16.4, 26.5, 6.4 and 28.0 μg. respectively of riboflavin per gram of dry tissue.

The fact that riboflavin is necessary in the diet of chickens not only to provide for the growth and health of the fowl, but also to assure that the muscle becomes a good source of riboflavin is important, since there is an increasing amount of

TABLE 1

The influence of diet on the riboflavin content of the tissue of chickens

TISSUE [1]	LOW RIBOFLAVIN DIET Riboflavin content of		HIGH RIBOFLAVIN DIET Riboflavin content of	
	Fresh tissue	Dry tissue	Fresh tissue	Dry tissue
	µg./gm.	*µg./gm.*	*µg./gm.*	*µg./gm.*
Liver	9.9	43	42.6	129
Liver	9.8	35	31.8	112
Liver	12.2	46	32.3	119
Liver	12.0	41	34.8	118
Liver	12.3	44	32.9	113
Liver	10.0	35	32.7	112
Liver	11.3	39	32.9	112
Liver	11.9	44	28.2	95
Liver	10.5	39	29.2	94
Liver	12.2	46	31.4	110
Liver average	11.2	41	32.9	111
Heart	4.3 [2]	19.4	18.4	71.2
Heart			20.4	80.4
Heart			16.7	63.0
Heart			18.4	72.3
Heart			19.5	72.6
Heart average	4.3	19.4	18.4	71.2
Gizzard	0.9	3.5	4.3	15.2
Gizzard	1.4	5.4	3.9	14.5
Gizzard	1.5	5.7	4.4	14.9
Gizzard	1.1	4.2	4.0	13.5
Gizzard	1.4	5.6	4.0	14.3
Gizzard average	1.3	4.9	4.1	14.5
Leg	1.0	4.3	4.6	18.8
Leg	1.1	4.7	4.1	15.6
Leg	1.0	4.8	7.4	29.3
Leg	0.7	3.5	5.6	21.3
Leg	0.8	3.2	6.0	24.0
Leg	1.0	4.4	3.4	14.5
Leg	1.0	4.3	2.4	10.1
Leg	1.0	4.7	3.1	13.0
Leg	1.1	3.8	3.2	13.3
Leg	1.0	4.7	2.5	10.6
Leg average	1.0	4.2	4.2	17.0
Breast	0.4	1.9	0.8	2.8
Breast	0.5	2.3	0.7	2.9
Breast	0.5	2.4	0.8	2.8
Breast	0.4	1.8	0.9	3.4
Breast	0.4	1.7	0.9	3.8
Breast average	0.4	2.0	0.8	3.1

[1] Each sample of a tissue is from one or more different chickens.

[2] Composite sample of hearts of five chickens.

evidence that riboflavin is necessary in human nutrition and that cases of ariboflavinosis often occur in the United States. This has been shown by Sebrell and Butler ('38, '39), Oden et al. ('39), Jolliffe and others ('39) and Kruse and associates ('40).

The effect of dietary riboflavin on the content of this vitamin in meat may not be limited to fowl but may also be important in certain other species such as swine, which are grown primarily for their meat. This question is one which appears worthy of further research. It has been reported by McElroy and Goss ('39) that dietary riboflavin is probably less important in the case of ruminants, as apparently bacteria in the rumen synthesize riboflavin.

SUMMARY

1. The amount of riboflavin in the diet has a marked effect on the riboflavin content of the tissues of chickens.

2. Of the tissues examined liver proved to be richest in riboflavin content; heart was next; gizzard and leg muscle were next and were about equal in value; breast muscle was poorest of all.

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THE COMPARATIVE ASSIMILATION OF FLUORINE BY GROWING RATS DURING CONTINUOUS AND INTERMITTENT DOSAGE [1,2]

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In studying the nutritional effects of nutrients, or the physiological effects of deleterious dietary constituents, by feeding experiments on animals, the customary procedure is to feed the nutrient or dietary constituent as a constant proportion of a suitably compounded ration. This is a systematic procedure that commends itself to most nutritionists and physiologists. However, in practical nutrition, at least in human nutrition, constancy in diet is the exception rather than the rule. It becomes a problem, therefore, whether the reaction of the body to the irregular intake of a dietary constituent is the same as the reaction to a regular intake. In particular, can the reaction to an irregular intake be predicted from the known results of a regular intake?

One possible cause of a quantitative difference in the physiological results of ingesting a given amount of a dietary constituent over a period of time, first, as a constant component of the diet, and, second, as an irregular component, is the interdependence in metabolism of the constituent in question

[1] This experiment was made possible by the donation of funds to the University of Illinois by the Aluminum Company of America and the Pennsylvania Salt Manufacturing Company.

[2] This investigation was conducted under the supervision of a Committee on the Physiological Effects of Spray Chemicals, appointed by the director of the Agricultural Experiment Station and consisting of the following members: H. H. Mitchell, W. A. Ruth, W. P. Flint and Julia P. Outhouse.

and one or more of the other components of the diet. Thus, the maximum effect of carbohydrate on protein metabolism is dependent upon the concurrent feeding of the two nutrients (Larson and Chaikoff, '37), while a demonstrable effect is dependent upon feeding them separately at intervals no greater than 4 hours in the case of canine subjects. Similarly, the favorable effects on experimental animals of a tryptophane supplement to a tryptophane-deficient diet are observed only when the supplement is fed concurrently with the basal diet, or at a time not too far removed from meal time (Berg and Rose, '29; Elman, '39).

The time relations in the interdependent action of two dietary components evidently depend upon the ability of the body to retain in its tissues in an unmetabolized form the component consumed first. This retaining ability will vary with the nature of the dietary component, and for the same component, it seems to vary also with the species of animal. Thus, the chick apparently stores vitamin A much less efficiently (and hence less readily) than the rat (Vermès, Lissot, Meunier and Raoul, '39). In the case of dietary constituents the physiological disposal of which is dependent upon other dietary components, it may be expected that the results of intermittent or irregular feeding may differ most from the results of continuous feeding when the storage ability of the organism is least; also, that, when a difference develops, the smaller degree of assimilation in the body will be associated with intermittent feeding. Furthermore, and for the same reasons, one might expect that, if two dietary constituents, the disposals of which in metabolism are to some extent interdependent, are consumed together, the metabolism of each will be modified to a different extent if the ratio of one to the other is changed.

When dietary components are stored in the body as a result of continuous feeding, the stores are subject to accretion only as the regime continues. But when storage occurs as a result of intermittent feeding, the stores would be subject both to accretion immediately following dosage, and to depletion by metabolism during the intervals between dosages. If the rate

of metabolism of the stored dietary component is greater than the rate of its metabolism when circulating in the body fluids and tissues, intermittent feeding again would be associated with less efficient utilization than would continuous feeding. A case in point is vitamin A, the reserves of which in the body are depleted at a rate directly proportional to their size (Baumann, Riising and Steenbock, '34), and, for the larger storages, at rates exceeding the day-to-day requirement. For this reason, large single doses of vitamin A or carotene are less effective nutritionally than small daily doses (Goss and Guilbert, '39). Apparently for a similar reason, massive doses of liver extract given at intervals of several months to patients with pernicious anemia may be less effective in prolonging the period of remission than small doses given at much shorter intervals of time (Strauss and Pohle, '40). The fact that the efficiency of calcium utilization by the growing rat is less the greater the storage of calcium in the body (Rottensten, '38) would seem also to have a bearing on this problem.

The consumption of sprayed fruits and vegetables in human nutrition is irregular, not only with reference to season, but undoubtedly also during periods when such foods are readily available. The ingestion of spray chemicals will also be irregular. Hence, their physiological effects may not be predictable, for the reasons just considered, from the results of feeding experiments involving the continuous administration of the chemical studied.

The purpose of the experiment to be described below is to study the comparative retention of fluorine in the spray chemical cryolite (synthetic) when offered to growing rats, first, as a constant component of the diet, and, second, as an irregular component of the diet; specifically, cryolite was added to the basal diet every third day throughout the feeding period. Comparative animals received the same total amount of fluorine. The concentration of fluorine in the total consumed food was purposely kept low, averaging only 7.34 p.p.m., in order that the results obtained would be more significant to the spray-residue problem.

EXPERIMENTAL METHODS

Twelve pairs of littermate rats were fed a basal ration consisting of the following ingredients, mixed in the indicated proportions: dried unextracted egg 30, dried extracted egg 7, ground yellow corn 55, dried yeast 3, fortified cod liver oil 2, fluorine-free salt mixture[3] 2, and calcium carbonate 1% respectively. This ration contained 1.4 p.p.m. of fluorine and was fed in equal amounts to pair mates. In addition to the basal diet, to which the rats had access continuously, or until the day's portion was consumed, one rat in each pair received daily 1 cc. of an aqueous solution of synthetic cryolite, containing 18 p.p.m. of fluorine, for each 3 gm. of food weighed out. The other rat in the pair received on every third day of feeding as much of the cryolite solution as its pair mate was given on that and the 2 preceding days. Thus, the total intakes of fluorine by pair mates were equalized every 3 days. The portions of the cryolite solution allotted to the rats were measured from a burette calibrated to 0.1 cc. and were mixed with the day's food. The solution was renewed at frequent intervals to insure against possible deterioration, and pair mates received the same volume of solution from each make.

After the sixth week of experimental feeding, the teeth of the rats were examined once a week for the appearance of striations, using a jeweler's lens with a magnification of 4 ×.

The experiment was terminated and the rats sacrificed generally at the end of 20 weeks. Three pairs were killed at the termination of 15 weeks, while with one pair the experiment had to be concluded after 88 days on account of the death of one of the rats, receiving continuous fluorine dosage, from a respiratory infection.

The rats were killed with ether and the empty weights and body lengths taken. The carcasses were then autoclaved and the flesh, bones and teeth separated and analyzed for fluorine by methods previously described (Lawrenz, Mitchell and Ruth, '39).

[3] Wesson.

EXPERIMENTAL RESULTS

Striations of the teeth were slow in appearing because of the low concentration of fluorine in the diets. Some rats failed to show striations even after 16 weeks of feeding, and among those developing definite striations no distinction could be made between those receiving a continuous dosage of fluorine and those receiving an intermittent dosage.

TABLE 1

Average data for all twelve pairs of experimental animals

	FLUORINE DOSAGE	
	Daily	Intermittent
Initial body weight, gm.	45	45
Total gain in weight, gm.	247	251
Final body length, mm.	232	233
Total food consumed, gm.	1264	1264
Number of food refusals	17	17
Total intake of fluorine, mg.	9.280	9.280
Feeding period, days	133	133
Final empty body weight, gm.	292	296
Weight of dry fat-free bone, gm.	13.6121	13.4315
Weight of dry teeth, gm.	0.4744	0.4694
Weight of soft tissues, gm.	267.5	269.7
Fluorine in bones, p.p.m.	224	212
Fluorine in bones, mg.	3.039	2.832
Fluorine in teeth, p.p.m.	116	112
Fluorine in teeth, mg.	0.055	0.053
Fluorine in soft tissues, p.p.m.	0.29	0.26
Fluorine in soft tissues, mg.	0.081	0.073
Total fluorine in carcass, mg.	3.175	2.957
Initial fluorine content, mg.	0.235	0.235
Total fluorine retained, mg.	2.940	2.722
Total fluorine retained, %	32.11	29.64

To conserve space, the growth data and the chemical data of the experiment are presented in average form in table 1. Each figure in the table is an average of twelve rats. Exclusive of the cryolite solutions, seventy-three samples were analyzed for fluorine.

The rats consumed an average of 1264 gm. of food and 9.280 mg. of fluorine, or 7.34 p.p.m. Nineteen per cent of the fluorine intake was, on the average, contained in the basal diet.

In an average period of 133 days the rats gained about 250 gm. in body weight, or 1.88 gm. per day.

Statistical analysis of the paired data of the experiment by the method of Student ('25) reveals no significant differences between continuous and intermittent feeding of fluorine with reference to gain in body weight, attained body length, weight of dry fat-free bone, dry weight of teeth or weight of soft tissue.

For the fluorine data, the statistics summarized in table 2, were obtained.

TABLE 2

The statistical significance of pair differences

ITEM	MEAN DIFFERENCE [1]	STANDARD DEVIATION OF DIFFERENCES	PROBABILITY
Fluorine in bones, p.p.m.	11.83	11.49	0.0029
Fluorine in bones, mg.	0.208	0.313	0.025
Fluorine in teeth, p.p.m.	4.33	8.49	0.060
Fluorine in teeth, mg.	0.00258	0.00545	0.073
Fluorine in soft tissues, p.p.m.	0.0283	0.1074	0.20
Fluorine in soft tissues, mg.	0.0080	0.0320	0.21
Total fluorine retained, mg.	0.217	0.237	0.0057

[1] A positive mean difference signifies that the mean for the continuous dosage rats exceeds the mean for the intermittent dosage rats.

All of the average differences between paired rats favored the rats receiving the continuous dosage of fluorine, i.e., for all samples and for the entire carcass, the average concentration of fluorine and the average total content of fluorine was greater for the continuous dosage rats. However, the average differences relating to the teeth and the soft tissues, both of which contained less than 5% of the fluorine in the carcass, were not significant statistically, as indicated by the probability values given in the last column of the table. These values express the probability on a scale of 1 that average differences as great or greater than those obtained, respectively, would have resulted from the operation of fortuitous factors only. If the probability is 0.03 or less, it may reasonably be neglected and the conclusion drawn that the corresponding average difference is the direct result, in part at

least, of the deliberately imposed difference in experimental treatment, namely, the method of administering fluorine.

From this statistical analysis, therefore, it may be concluded that the continuous feeding of fluorine has resulted in a greater retention of the element in the carcasses of the rats, and specifically in the bones, than the intermittent feeding of the same amount of fluorine. Under the conditions of this experiment, 7.4% less fluorine was retained when administered intermittently (every third day) than when administered daily.

The application of this finding to the spray residue problem would seem to be that the physiological effects of cryolite residues on sprayed fruits or vegetables should be somewhat less under conditions of practical nutrition than would be expected from experiments involving constant daily dosage.

CONCLUSIONS

1. The continuous administration of fluorine as synthetic cryolite to growing animals results in a greater retention of fluorine in the bones, and possibly in the teeth, than the intermittent administration of the same quantity of the element.

2. Hence, in practical human nutrition, which involves variable and intermittent consumption of foods, the physiological effects of a given dosage of fluorine in spray residues on fruits or vegetables may be expected to be somewhat less than that predicted from experiments involving the continuous administration of fluorine.

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AVAILABILITY TO WHITE RATS OF PHOSPHORUS IN SOYBEAN AND RED CLOVER HAYS

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THE PROBLEM

This paper represents a continuation of the studies already published ('40) comparing the availability to white rats of the phosphorus of a low phosphorus hay and of a high phosphorus hay. The types of hay used in this work were soybean and red clover.

EXPERIMENTAL PROCEDURE

Rapidly-growing white rats, 30 to 31 days old, and weighing from 42 to 71 gm. were used as experimental animals. Five male rats were put on each diet in the soybean series; and five males and five females, in the red clover series.

Phosphorus analyses of the hays used in the experimental diets were as follows: the low phosphorus soybean hay, 0.12% (0.27% P_2O_5); the high phosphorus soybean hay, 0.21% (0.48% P_2O_5); the low phosphorus red clover, 0.12% (0.27% P_2O_5); and the high phosphorus red clover, 0.31% (0.71% P_2O_5).

As indicated in tables 1 and 2, the diets were so constructed that energy, fiber, mineral, and vitamin contents were the same for all animals in a given series, insofar as was possible, and all essential nutrients were present in adequate amounts with the exception of phosphorus. The amount of phosphorus for each diet was approximately 0.16% since, as previously

TABLE 1

Composition of experimental diets

COMPONENT	SOYBEAN HAY DIET			RED CLOVER HAY DIET		
	Control	Low P hay	High P hay	Control	Low P hay	High P hay
	%	%	%	%	%	%
Hay	0	67	39	0	69	26
Lactalbumin	18	7	13	18	11	15
Starch + yeast extract	45	12	22	49	6	34
Agar	23	0	12	19	0	11
Salt mixture[1]	4	4	4	4	4	4
Butterfat	8	8	8	8	8	8
Cod liver oil	2	2	2	2	2	2

[1] Osborne and Mendel salt mixture modified to supply 0.16% phosphorus for each diet and equal amounts of calcium for each diet in a given series. Copper sulfate has also been added to the salt mixture.

TABLE 2

Distribution of foodstuffs among the various constituents of the soybean and red clover diets

DIETARY FACTOR		SOYBEAN HAY DIETS			RED CLOVER HAY DIETS		
		Control	Low P hay	High P hay	Control	Low P hay	High P hay
		%	%	%	%	%	%
Protein:	Hay	0	11	5	0	7	3
	Lactalbumin	18	7	13	18	11	15
	Total	18	18	18	18	18	18
"N-free extract":	Hay	0	22	15	0	28	10
	Starch	45	12	22	49	6	34
	Total	45	34	37	49	34	44
Fat:	Butter	8	8	8	8	8	8
	Cod liver oil	2	2	2	2	2	2
Fiber:	Hay	0	23	11	0	19	8
	Agar	23	0	12	19	0	11
	Total	23	23	23	19	19	19
Calcium:	Hay	0	0.710	0.386	0	0.959	0.263
	Lactalbumin	0.036	0.014	0.026	0.036	0.022	0.030
	$CaCO_3$	0.653	0	0.301	0.868	0	0.686
	Total	0.689	0.724	0.713	0.904	0.981	0.979
Phosphorus:	Hay	0	0.080	0.080	0	0.080	0.080
	Lactalbumin	0.027	0.011	0.020	0.027	0.017	0.023
	H_3PO_4	0.118	0.077	0.062	0.132	0.066	0.058
	Total	0.145	0.168	0.162	0.159	0.163	0.161
Ca/P ratios		4.8	4.3	4.4	5.7	6.0	6.1

shown, this furnishes about the minimal amount required for normal growth and bone development. The amount of calcium in each diet of a given series was the same, and was determined by the diet which originally contained the most. Since the red clover contained a higher percentage of calcium than the soybean hay, the Ca/P ratio in the red clover diets was higher than that in the soybean diets, namely, 5.7 : 1 and 4.8 : 1, respectively.

The triplicate feeding method employed in the previous studies with lespedeza sericea and alfalfa was used here. Of three animals of the same sex, litter, and approximately the same weight, one animal was put on the control diet, and one each on the experimental diets being compared. The food consumption of the trio was kept uniform.

The animals were started on the experimental diets at 30 to 31 days of age and were killed at 60 to 61 days of age. Rate of growth and phosphorus retentions were used as criteria in comparing the effects of the different diets on the rats. The rats were ashed at approximately 550°C. and analyzed for phosphorus by the volumetric method essentially as approved by the Association of Official Agricultural Chemists ('35).

RESULTS

Table 3 summarizes the average results obtained for all animals. These results substantiate those obtained under similar experimental conditions using lespedeza and alfalfa hays. The rats, male or female, receiving one-half their total phosphorus from a low phosphorus hay, either soybean or red clover, were conspicuously less thrifty animals than either the control animals or those deriving one-half the phosphorus from a hay high in phosphorus content whether judged on the basis of gain in weight, body length, or the amounts of phosphorus retained in the body. In the case of the soybean hay, the rats fed the control, low phosphorus hay diet, and the high phosphorus hay diet gained on the average 68, 31, and 46 gm., respectively, during the 30-day experimental period; and stored in their bodies, on the average, 0.638, 0.479,

TABLE 3

Summary of average results for experimental animals in growth and phosphorus intake and retention including summaries from variance analyses for phosphorus content of rat at 60–61 days of age

EXPERIMENTAL GROUP	NO. OF CASES	WEIGHT			LENGTH OF TRUNK	TOTAL FECES DRY WT.	FOOD CONSUMPTION WITH A.D.[1]	TOTAL P IN FOOD WITH A.D.	P IN RAT AT 60–61 DAYS WITH A.D.	P IN RAT AT 30–31 DAYS WITH A.D.	P STORED FROM FOOD WITH A.D.	P FROM FOOD
		Initial net	Final net	Gain net								
		gm.	*gm.*	*gm.*	*cm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	%
Triplicate feeding												
Soybean—males												
Control	5	55	123	68	15.4	54	238 ± 16	0.345 ± 0.023	0.638 ± 0.027	0.293 ± 0.019	0.345 ± 0.020	100
Low P	5	55	86	31	14.0	114	239 ± 17	0.401 ± 0.026	0.479 ± 0.025	0.292 ± 0.012	0.186 ± 0.016	46
High P	5	56	102	46	14.6	98	239 ± 19	0.376 ± 0.036	0.540 ± 0.029	0.297 ± 0.008	0.243 ± 0.021	65
F value for diets									65.05[2]			
Significant difference									0.032			
Red clover—males												
Control	5	51	105	54	15.4	57	239 ± 24	0.379 ± 0.038	0.535 ± 0.041	0.272 ± 0.042	0.263 ± 0.024	69
Low P	5	51	63	12	13.4	121	239 ± 23	0.389 ± 0.038	0.372 ± 0.047	0.271 ± 0.031	0.101 ± 0.026	26
High P	5	52	92	40	14.6	91	241 ± 24	0.387 ± 0.039	0.492 ± 0.044	0.274 ± 0.031	0.218 ± 0.024	56
F value for diets									79.23[2]			
Significant difference									0.031			
Red clover—females												
Control	5	52	89	37	14.6	51	217 ± 19	0.345 ± 0.030	0.498 ± 0.036	0.276 ± 0.021	0.223 ± 0.032	65
Low P	5	52	58	6	13.0	113	222 ± 19	0.360 ± 0.030	0.404 ± 0.029	0.277 ± 0.016	0.127 ± 0.014	35
High P	5	52	80	28	14.2	84	222 ± 19	0.357 ± 0.031	0.483 ± 0.031	0.275 ± 0.018	0.208 ± 0.036	58
F value for diets									23.69[2]			
Significant difference									0.034			

[1] A.D. = average deviation.

[2] F value exceeds the 1% point.

and 0.540 gm. phosphorus, respectively. As shown in table 3, the control rats stored 100% of the readily available phosphorus furnished in the food. Those fed the low phosphorus hay diet retained less than 50% of the dietary phosphorus, all of which could have been obtained from the phosphorus of the salt mixture. The animals receiving the high phosphorus hay diet retained more than 50% of the food phosphorus and therefore must have obtained some of it from the hay. It is to be noted that the amounts of feces eliminated on each diet are in inverse ratio to the gains in weight and the phosphorus retained by the animals. Similar relationships hold for the rats, male and female, in the red clover series.

A variance analysis of the data gives evidence that the differences in phosphorus retention on the different diets within a given series are highly significant. The average difference in phosphorus retention of the animals on the low phosphorus hay diets and those on the high phosphorus hay diets in each series is larger than the amount required for significance.

It is of interest to point out that the gains in weight of the animals and the percentages of phosphorus of the food stored are considerably less for each corresponding diet in the red clover than in the soybean series. Considering only the males, it will be seen that it so happened that the food consumptions were nearly identical for the two series. The only obvious difference between the diets in the two series, especially in relation to the control diets, is a difference in Ca/P ratio which is more unfavorable in the red clover diets than in the soybean diets. Applying "Student's" t-test, according to Goulden ('39), to the average difference of 0.103 gm. in the amount of phosphorus at 60 days between the control rats in the red clover series and the soybean series, it is found that in only two cases in 100 would a difference of that size occur by chance.

It therefore seemed worth while to collect all the available data concerning Ca/P ratios obtained with the animals used in the previous work on lespedeza and alfalfa hays. Considering only the controls in any series of animals, which

were thus fed practically the same diet except for the amount of calcium included in the salt mixture, there seems to be a relationship between Ca/P ratio and average amount of phosphorus stored in the rat at 60 days. It is to be remembered that in all cases the amount of phosphorus fed was at a minimal level.

The data in table 4 indicate that as the Ca/P ratio increases, the food consumption and the amount of phosphorus stored in the rat decrease. These results are in agreement both with the work of Bethke, Kick and Wilder ('32) who state that increasing the Ca/P ratio from 1 to 5 causes progressive decrease in growth, bone ash, and percentage inorganic blood phosphorus in the rat; and with Brown, Shohl, Chapman,

TABLE 4

Comparison of amount of phosphorus stored on the basis of Ca/P ratios

NO. OF RATS	Ca/P RATIO	FOOD CONSUMPTION	P IN RAT AT 60 DAYS
		gm.	*gm.*
4	1.5	349 ± 17	0.886 ± 0.013
33	3.3	309 ± 40	0.700 ± 0.105
15	4.8	251 ± 36	0.650 ± 0.069
6	5.7	232 ± 25	0.521 ± 0.053

Rose and Sauerwein ('32) who find that at a given level of Ca (or P), increasing the ratio of Ca/P intensifies the degree of rickets in rats. They emphasize that both the level and the ratio are necessary adequately to characterize the ricketogenic properties of a diet. The question of Ca/P relationships is emphasized not so much in connection with the experimental work presented in this paper, since Ca/P ratios were carefully controlled in animals being compared directly, but to point out the practical implication which follows from the fact, as reported by Weathers ('38), that when hays which are low in phosphorus are compared with high phosphorus hays of the same type, they seem to have a higher Ca/P ratio which in itself may affect adversely the nutritional quality of a low phosphorus hay.

SUMMARY OF RESULTS

1. When the experimental diets contain phosphorus at a minimal level of adequacy and all other nutrients at optimal levels and essentially the same for all diets, the phosphorus of a low phosphorus soybean or red clover hay is less available to the rat for growth and bone development than the phosphorus of a high phosphorus hay of the same type.

2. Evidence has been presented indicating that for the rat, with the total phosphorus of the diet at a minimal level, an increase in Ca/P ratio is accompanied by a decrease in food consumption and in the amount of phosphorus stored in the animal's body.

CONCLUSION

The present paper extends to soybean and red clover hay the results concerning the availability of phosphorus in lespedeza sericea and alfalfa. For four different kinds of leguminous hay, it has now been shown that rats consuming a relatively large amount of a low phosphorus hay to supply the minimal phosphorus requirement grow less and store less phosphorus over a given time than rats receiving the same amount of phosphorus from a smaller amount of a high phosphorus hay of the same type. The explanation for this difference in availability of the phosphorus seems to be related to the fact that there is a marked increase in the amount of feces eliminated on the diet containing the low phosphorus hay as compared to the amount eliminated on the diet with the high phosphorus hay. The possibility is recognized that the differences in phosphorus content of the hay may accompany other differences in quality of the hay not brought out by routine analysis, such as in the fraction designated as "nitrogen-free extract."

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THE PREVENTION AND CURE OF NUTRITIONAL MUSCULAR DYSTROPHY IN THE RABBIT BY ALPHA-TOCOPHEROL IN THE ABSENCE OF A WATER-SOLUBLE FACTOR

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Morgulis and his co-workers (Morgulis and Spencer, '36; Morgulis, Wilder and Eppstein, '38; Morgulis, '38 and '38–'39) have reported that nutritional muscular dystrophy in the rabbit is a multiple deficiency disease produced by the absence of either a water-soluble or a fat-soluble factor. We have recently identified the fat-soluble antidystrophy factor as alpha-tocopherol (Mackenzie and McCollum, '39, '40). Support for the dual nature of the deficiency may be found in the early failure of Goettsch and Pappenheimer ('31) to prevent the disease with wheat germ oil.

Confirmation of the observation of Morgulis et al. that the absence of either of two factors results in the same reaction of a specific tissue would be of great theoretical as well as practical importance. A similar interrelationship in other species has not been excluded, since the diets used to produce vitamin E deficiency have included a source of the postulated water-soluble factor, namely, yeast. Consequently the conclusion of Goettsch and Ritzmann ('39) that the rat can dispense with the water-soluble factor was not valid.

Attempts by us during the past year to demonstrate that a water-soluble factor is essential for the integrity of the skeletal muscles of rabbits fed the diet employed by Morgulis have failed. We have, however, extended our observations on the

alpha-tocopherol requirements of the rabbit, and obtained information on the production of chronic dystrophy.

METHODS

Animals and their care. The methods employed both for diagnosing dystrophy and detecting a response to a positive supplement, and for the housing and care of the animals, have been previously described (Mackenzie and McCollum, '40). Attention is called to the practice of withholding food for several hours before and after administering supplements to prevent their mixture with food in the stomach. Supplements were fed by mouth from syringes equipped with long blunt needles.

Young rabbits, weighing from 300 to 400 gm., were obtained from the colony of the Department of Immunology of the School of Hygiene and Public Health. This stock is snuffles-free and the number of deaths from coccidiosis is low. The creatine excretion of these animals is relatively constant, and does not exceed 15 mg. daily or 10 mg. per kilogram of body weight. An appreciable increase has been invariably accompanied by muscle lesions. The necessity for establishing these facts was recently demonstrated when we obtained from a dealer a group of rabbits exhibiting a high and fluctuating creatinuria.

Diets. The basal diet used by us was the one generally employed by Morgulis and his co-workers, diet 13 of Goettsch and Pappenheimer ('31). In most of our experiments this diet was modified by increasing the U.S.P. hydrated ferric chloride from a level of 1% to 1.67%. Consequently the level of anhydrous ferric chloride was 1%. The modified diet 13 has been designated as no. 133.

Supplements. Petroleum ether-extracted wheat germ was prepared by extracting fresh wheat germ [1] in the Soxhlet

[1] Wheat germ was obtained from the Russell Miller Milling Company, Minneapolis, Minnesota.

apparatus or in a continuous extractor with C. P. petroleum ether (B. P. 35 to 60°C.) for 24 hours. Ethyl ether-extracted wheat germ was prepared by extracting wheat germ in the continuous extractor with cool U.S.P. ethyl ether for 24 hours.

In preparing a concentrate of the water-soluble factor the procedure of Morgulis was closely followed. Petroleum ether-extracted wheat germ was covered with 70% alcohol, stirred vigorously for 2 hours and allowed to stand overnight at room temperature. The extract was filtered off and the extraction repeated until the filtrate was colorless. The filtrates were combined and the alcohol removed by distillation under reduced pressure from a water bath at 40°C. The precipitate formed was filtered off and discarded. Benzoic acid was added to the filtrate as a preservative. Extracts were prepared fresh weekly and stored in the refrigerator in a glass stoppered flask.

An alternative procedure was based on the extractability of the water-soluble factor by acetone but not by ether, as reported by Morgulis, Wilder and Eppstein ('38). Ethyl ether-extracted wheat germ was extracted with warm C. P. acetone in the Soxhlet apparatus for 24 hours. During this time a white precipitate was formed in the extraction flasks. The acetone was decanted and distilled off at the water pump. The residue was taken up in water as was the precipitate in the extraction flasks and the two combined. Benzoic acid was added as a preservative.

The petroleum ether extract of wheat germ was used as a source of wheat germ oil. The petroleum ether solution was filtered and the solvent removed at the water pump. The oil was stored in the refrigerator.

Synthetic alpha-tocopherol [2] was used as the source of vitamin E. Ethyl laurate solutions containing 20 and 50 mg. per cubic centimeter were prepared at least every 2 weeks and kept in glass stoppered bottles in the refrigerator.

[2] We are indebted to Merck and Company, Inc., for the supply of alpha-tocopherol.

EXPERIMENTAL

Increasing the ferric chloride[3] content of diet 13 from 10 to 16.7 gm. per kilogram resulted in the earlier death of young animals fed the unsupplemented diet as shown in table 1. The symptoms of dystrophy were frequently attended and sometimes obscured by diarrhea and respiratory infections. These complications considerably shortened the survival time of many of the sick animals. While the more drastic action of diet 133 (diet 13 with increased ferric chloride) was probably due in part to the increased destruction of vitamin E, other modes of action of the salt, for example on mineral metabolism, cannot be eliminated. As will be shown later, however, such

TABLE 1

The effect of increasing the ferric chloride[1] content of diet 13 on the survival time of young rabbits

NUMBER OF RABBITS	AVERAGE WEIGHT	DIET	FERRIC CHLORIDE	PER CENT DEAD				
				1 week	2 weeks	3 weeks	4 weeks	5 weeks
	gm.		*per cent*					
8	380	13	1.0	0	0	0	38	50
12	390	133	1.67	0	0	34	75	84

[1] The hydrated salt is referred to.

effects must have been entirely dependent on the development of vitamin E deficiency.

The addition of alpha-tocopherol to diet 133. Sixteen young rabbits were placed on diet 133 and fed alpha-tocopherol at levels of 3 mg., 1 mg., and 0.5 mg. daily, 6 days a week. Five more animals were fed the diet without alpha-tocopherol. Administration of the supplement and care of the animals have been described in the section entitled Methods. The diagnosis of dystrophy was based either on an increased excretion of creatine or the presence of lesions in the thigh muscles.

It is apparent from table 2 that the 3 mg. level of the vitamin afforded complete protection against muscular dystro-

[3] Here and throughout the remainder of the paper ferric chloride refers to the hydrated salt.

TABLE 2

The preventive action of alpha-tocopherol fed to rabbits reared and maintained on dystrophy-producing diet 133

ANIMAL	SEX	DAILY DOSE OF α-t [1]	INITIAL WEIGHT	TIME TO ABNORMAL CREATINURIA	MAXIMUM WEIGHT	LOSS IN WEIGHT	DURATION OF EXPERIMENT	FIRST PHYSICAL SYMPTOMS	MICROSCOPIC LESIONS [3]	COMMENTS
		mg.	*gm.*	*weeks* [2]	*gm.*	*gm.*	*weeks*	*weeks*		
52	♀	3	350		1750	0	12		......	Killed
46	♀	3	290		1730	0	12		......	Killed
55	♂	3	480		2380	0	14		......	Killed
53	♂	3	440		2550	0	17		......	Killed
66H	♀	3	850	..	2880	0	20		......	Killed
67H	♂	5	950		3570	0	21		......	Killed
50	♂	3	400		2540	0	24		......	Killed
22B	♂	1	470	12	2310	0	18		± [4]	Killed
22D	♀	1	290	12	1860	0	17		+	Killed
25	♀	1	300		2680	120	31		+±	Killed
22C	♀	0.5	480		1610	60	8	8	+++	Died. Diarrhea
71	♀	0.5	330	7	1710	50	9	8	++	Killed
74	♂	0.5	370	7	1900	0	12			Discontinued
62		0.5	300	6	1760	100	12		++	Died. Diarrhea
22A	♂	0.5	500	9	2490	0	16			Discontinued
72	♂	0.5	420	<11	3170	0	20		+±	Killed
70	♂	0	320	3	980	220	3½	3½	++++	Died. Respiratory infection
73		0	305	3	850	170	4	3½	++++	Killed. Diarrhea
77	♀	0	430	2½	940	110	3	3	++++	Killed
79	♂	0	400	3	970	200	4	3	++++	Died. Diarrhea
81		0	430	2	950	180	3	2½	+++	Killed. Respiratory infection

[1] Alpha-tocopherol given 6 days a week.

[2] The week during which the daily creatine excretion reached 20 mg. has been selected as the beginning of definitely abnormal creatinuria.

[3] Biceps femoris and vastus lateralis examined.

[4] Only one or two lesions (accumulations of nuclei) per section. Creatine excretion at 16 weeks had fallen to 4 to 5 mg. per kilogram of body weight.

phy. The 1 mg. level of alpha-tocopherol did not suffice to prevent symptoms of dystrophy indefinitely, though in the case of rabbit 22 B the slightly abnormal creatinuria occurring at the twelfth week was transitory, declining from 4 to 5 mg. per kilogram of body weight at the sixteenth to eighteenth week. At the eighteenth week the microscopic lesions of the muscles were confined to one or two masses of nuclei per section. In the case of the other two animals the lesions were more extensive. However, rabbit no. 25 was carried for 31 weeks. None of these animals exhibited physical symptoms.

Considerable variation was observed in the rabbits fed 0.5 mg. of alpha-tocopherol, as shown in table 2. One animal died at 8 weeks and one at 12 weeks (both had diarrhea), while two others were alive at 16 and 20 weeks. Rabbits 22 C and 71 were the only ones to show physical symptoms of dystrophy during the experiment. The 0.5 mg. level of alpha-tocopherol postponed the rise of urinary creatine from 3 weeks (2.0 to 3.5 for unsupplemented animals) to 7 (6 to 9) weeks. This in turn is earlier than the time of creatine rise found for two of the rabbits on the 1.0 mg. level.

From this experiment it was concluded that the administration of sufficiently large amounts of alpha-tocopherol to rabbits on diet 133 prevented dystrophy. The possibility that we were working with a special strain of animals that did not require the postulated water-soluble factor of Morgulis, and that this factor was essential for other rabbits, was partially eliminated by including in the experiment two rabbits, 66H and 67H, that were unrelated to our main stock. We next turned our attention to the question of a quantitative relationship between alpha-tocopherol and a water-soluble factor.

The effect of defatted wheat germ and its extracts on chronic dystrophy. Chronic dystrophy was produced in four rabbits on diet 133 through the administration of subminimal doses of alpha-tocopherol. The defatted wheat germ supplements were mixed in the basal diet daily at a 10% level. Higher levels resulted in reduced and incomplete food consumption. Extracts of defatted germ were given by mouth as described

in the section headed Methods. The results of this experiment as shown by table 3 were negative. The ability of the animals to respond to positive supplements was confirmed by feeding alpha-tocopherol or wheat germ oil at the end of the experiment.

TABLE 3

The effect of defatted wheat germ and its extracts on the creatinuria of rabbits with chronic dystrophy on diet 133 plus subminimal levels of alpha-tocopherol

ANIMAL	SEX	ALPHA-TOCOPHEROL DAILY [1]	DAYS ON DIET	DAILY SUPPLEMENTS	DAYS OF TREATMENT	AVERAGE WEIGHTS, GM.	AVERAGE DAILY CREATININE. MG. PER KGM. OF WEIGHT [2]	AVERAGE DAILY CREATINE. MG. PER KGM. OF WEIGHT [2]
		mg.						
102	♂	0.5	69	E.E.W.G. = 6 gm. Acet. E ≎ 20 gm.	10	2130	28	10
		0.5	82		8	2320	30	19
		0.5	90	Al. E ≎ 20 gm.	8	2420	31	15
		0.5	98	P.E.W.G. = 6 gm. Al. E ≎ 14 gm.	14	2450	27	18
		0	113	Wheat germ oil = 0.7 cc.	14	2500	23	4
106	♀	0.5	35		15	1270	23	13
		0.5	50		13	1470	21	20
		0.5	63	Acet. E ≎ 20 gm.	11	1620	26	20
		0.5	74	P.E.W.G. = 5 gm. Al. E ≎ 20 gm.	9	1700	19	18
104	♂	0.5	52		11	1710	30	18
		0.5	63	P.E.W.G. = 6 gm. Al. E ≎ 20 gm.	14	1890	26	20
		0.5	77		7	1900	25	17
		0	84	Alpha-tocopherol = 3 mg.	12	2160	26	4
105	♀	1.0	82		10	1630	37	12
		1.0	92		8	1710	36	15
		1.0	100	Al. E ≎ 20 gm.	8	1800	32	16
		1.0	108	P.E.W.G. = 5 gm. Al. E ≎ 20 gm.	10	1820	30	19
		0	119	Alpha-tocopherol = 3 mg.	13	1860	27	5

E.E.W.G. = Ethyl ether-extracted wheat germ.

P.E.W.G. = Petroleum ether-extracted wheat germ.

Acet. E. = Acetone extract of ethyl ether-extracted wheat germ.

Al. E. = 70% alcohol extract of petroleum ether-extracted wheat germ.

[1] Alpha-tocopherol given 6 days a week.

[2] Creatine and creatinine determinations made on 1 or 2 day collections of urine.

The response elicited by vitamin E was not dependent on the previous administration of sources of the postulated water-soluble factor. This was demonstrated by treating three animals that developed chronic dystrophy on diet 133 (in the absence of a source of the water-soluble factor) with 3 mg. of alpha-tocopherol daily. In each case the abnormal creatinuria fell rapidly to the normal level.

This experiment failed to disclose a quantitative relationship between vitamin E and a water-soluble factor, and demonstrated the cure of chronic dystrophy with vitamin E alone. Nevertheless a third possibility existed, namely, that alpha-tocopherol alone would not suffice to cure acute dystrophy because of the development of some secondary deficiency induced by low food consumption, digestive disturbances or extensive muscle damage.

The effect of alpha-tocopherol on acute dystrophy. Since, as was previously mentioned, many of the animals on unsupplemented diet 133 developed diarrhea and respiratory infections and died soon after the first signs of illness, we reverted to diet 13 in this experiment. This also enabled us to compare the results obtained with those reported in our first paper (Mackenzie and McCollum, '40) on the curative action of alpha-tocopherol administered to animals on diet 13 plus defatted wheat germ.

Seven young rabbits were placed on diet 13 and two on diet 13 plus 10% of ethyl-ether-extracted wheat germ. The average weight for the group was 330 gm. The daily creatine excretion reached 20 mg. in 21 (16 to 27) days. At the time of treatment three of the animals had stage II dystrophy and six stage III dystrophy (Mackenzie and McCollum, '40). The creatine excretion had reached an average level of 55 (40 to 80) mg. per day, or an average of 62 mg. per kilogram of body weight. Four of the animals had ceased eating. The administration of 3 mg. of alpha-tocopherol daily for 5 days resulted in a prompt and typical response in all cases. The creatine excretion dropped appreciably in from 1 to 3 days

and soon returned to the normal level. The physical symptoms disappeared completely and growth was resumed.

Four of the cured animals were continued on diet 13 without vitamin E. The daily creatine excretion once more rose to 20 mg. in from 23 to 31 days after the first dose of alpha-tocopherol. The use of smaller animals in this experiment probably accounts in part for the longer period required for the rise in creatine as contrasted with our first report of 15 to 20 days (Mackenzie and McCollum, '40). Three of the animals continued on diet 13 were allowed to develop acute dystrophy and were again cured with alpha-tocopherol.

The results reported in this section demonstrated that in our hands, and with our method of administering supplements, acute dystrophy produced on diet 13 could be consistently and repeatedly cured with alpha-tocopherol alone. It still remained for us to obtain evidence that our diets were not contaminated with the water-soluble factor reported by Morgulis and his co-workers.

The effect of incorporating wheat germ oil in diet 133. Morgulis ('38–'39) in his most recent publication states that in preventive treatments the supplements are incorporated in the diet and that in curative treatments the supplements are given to prostrated animals by pipette, but that as soon as the animals are on their feet and able to eat, the supplements are incorporated in the diet as in the case of less severely dystrophic animals. In other words, if wheat germ oil is added to the diet as a source of the fat-soluble factor the rabbits should develop dystrophy as a consequence of the water-soluble factor deficiency unless this factor is also present in the diet. In preventive experiments this is the criterion for the existence of the water-soluble factor.

Wheat germ oil prepared from a petroleum ether extract of wheat germ was mixed in diet 133 in amounts sufficient to insure the consumption of approximately 2 cc. of oil per day. This mixture was prepared in amounts sufficient to last for 3 or 4 days following the procedure of Morgulis ('38, p. 80). The oil was potent at a level of 1 cc. per day when administered

to rabbits rendered dystrophic on diet 133 plus 10% of defatted wheat germ.

The result of mixing wheat germ oil in diet 133 is shown in table 4. The first sign of dystrophy, a rise in the creatine excretion, was observed in from 35 to 40 days, and gross symptoms occurred soon thereafter. This confirmation of the observation of Morgulis ('38, '38–'39) indicated that our basal diet was deficient in the suggested water-soluble factor.

TABLE 4

Effect of the addition of wheat germ oil to diet 133. Animals 80 and 83 received in addition 10% of defatted wheat germ in the diet from the thirty-seventh to seventy-seventh day

Animal	75	76 [1]	78	84	80	83
Sex	♀	♀		♂	♂	♂
Wheat germ oil daily, cc.	2.0	1.7	1.9	1.8	1.9	2.0
Initial weight, gm.	300	320	360	360	370	420
Time to abnormal creatinuria,[2] days	40	35	35	39		
Maximum creatine,[3] mg./kgm.	50	66	80	50	8	10
Maximum weight, gm.	1610	1330	1350	1170	2060	2060
Loss in weight, gm.	160	220	230	200		
Duration of experiment, days	62	40	38	38	98	98
First physical symptoms, days	53	38	35	39		

[1] Animal 76 was cured of dystrophy at the end of the experiment by omitting the oil from the diet and administering 1 cc. per day by mouth.

[2] The day on which the creatine excretion reached 20 mg. has been selected as the beginning of definitely abnormal creatinuria.

[3] Expressed as milligrams per kilogram of body weight per day.

Two additional animals on diet 133 plus wheat germ oil, that showed no signs of dystrophy on the thirty-seventh day of the experiment, were supplemented with 10% of petroleum ether-extracted wheat germ at that time (see table 4). The defatted germ was mixed in the diet and the oil then added as described above. By the seventy-seventh day these animals had shown no signs of dystrophy and the germ and oil were both discontinued. The animals were continued for 3 weeks on diet 133 at which time the creatine excretion for no. 80 and no. 83 was 3 and 6 mg. per kilogram of body weight respectively. These results suggest that the defatted wheat germ

protected the vitamin E in the oil from destruction by the diet.

Vitamin B_6. Although all of our attempts to demonstrate the existence of a water-soluble antidystrophy factor had failed we were prompted by the report of Antopol and Schotland ('40) on the beneficial results obtained with vitamin B_6 in cases of human pseudo-hypertrophic muscular dystrophy to try the effect of the vitamin on rabbits. Three rabbits on diet 13 were given 5 mg. of vitamin B_6 hydrochloride [4] daily by mouth at about 10 days before a rise in urinary creatine was to be expected. In each case severe dystrophy developed. An additional animal with a high creatinuria received a total of 400 mg. of vitamin B_6 subcutaneously over a 12-day period. The creatine remained elevated and the rabbit developed severe physical symptoms of dystrophy during the treatment. While this experiment did not eliminate a transitory effect of B_6 on the dystrophic rabbit muscle, it showed that B_6 in large amounts cannot replace vitamin E in preventing or curing the disease.

DISCUSSION

We have attempted to confirm the observation of Morgulis and co-workers (Morgulis and Spencer, '36; Morgulis, Wilder and Eppstein, '38; Morgulis, '38 and '38–'39) that rabbits reared on diet 13 of Goettsch and Pappenheimer ('31) require for the structural integrity of their skeletal muscles two vitamins, the absence of either of which results in the necrosis of these muscles. Our experiments indicate that, when proper precautions are taken to prevent the destruction of vitamin E by the diet, the dystrophy may be prevented or cured by alpha-tocopherol alone. Furthermore, we have been unable to demonstrate a quantitative or synergistic relationship between alpha-tocopherol and a water-soluble factor in the cure of chronic dystrophy produced by feeding subminimal doses of alpha-tocopherol.

[4] We are indebted to Merck and Company, Inc., for the supply of vitamin B_6 hydrochloride.

The fact that the addition of a potent wheat germ oil to the dystrophy-producing diet failed to prevent the disease indicates that our diet was, according to the criteria employed by Morgulis, deficient in the postulated water-soluble factor. A plausible explanation for the discrepancies between our results and those obtained by Morgulis is furnished by the hypothesis that defatted wheat germ contains one or more substances protecting vitamin E from destruction by the rancid diet. Some evidence that this may be the case has been presented. This question is being made the subject of further investigation.

The failure of Goettsch and Pappenheimer ('31) to prevent dystrophy in rabbits even though wheat germ oil was administered by mouth was probably due to the fact that their animals had continual access to the diet. We have observed (unpublished data) that rabbits having continual access to an E-deficient diet and receiving ample amounts of alpha-tocopherol by mouth become dystrophic when cod liver oil is added to the diet.

We have fully confirmed our earlier observations (Mackenzie and McCollum, '40) on the prevention and cure of nutritional muscular dystrophy in the rabbit with alpha-tocopherol. The level of the vitamin necessary to prevent muscle lesions in growing rabbits lies between 6 and 18 mg. per week though 3 mg. per week will greatly prolong life. On the latter dosage animals may grow for months without exhibiting physical symptoms. However, there is a definite creatinuria, and microscopic lesions are found at autopsy. Taken in conjunction with the duration of the effect of the 15 mg. curative dose, the daily requirement of the rabbit for alpha-tocopherol may be again roughly fixed at 0.6 to 1.0 mg. per kilogram of body weight. An appreciable gap exists between this figure, representing the amount necessary to prevent muscle lesions, and the intake that will suffice to maintain normal growth and prevent gross symptoms.

It thus appears that a number of muscle fibers can be damaged without seriously affecting the health of the animal

(though its activity is possibly reduced) and that the number of fibers damaged is inversely proportional to the vitamin E intake. The data in tables 2 and 3 indicate that the quantitative aspect of such a relationship varies between individuals, possibly as a result of differences in the absorption and transportation of the vitamin and the degree of muscular activity.

The relative constancy of the creatinuria from week to week in cases of chronic dystrophy suggests that this relationship is maintained by an equilibrium between degenerative and regenerative processes. Histological examinations of the muscles of animals with chronic dystrophy tend to confirm this hypothesis. All of these considerations support the theory previously advanced (Mackenzie and McCollum, '40) that vitamin E is directly involved in the metabolism of the rabbit's voluntary muscles. The comparative study of the distribution of vitamin E in the tissues of several species and in muscles showing different degrees of injury is planned.

The production of chronic dystrophy through the administration of from 3 to 6 mg. of alpha-tocopherol per week furnishes excellent material for studying the metabolism of the dystrophic animal in the absence of complications arising from loss of weight, reduced food consumption or secondary pathological conditions.

SUMMARY

1. We have been unable to confirm the report of Morgulis and co-workers that rabbits fed diet 13 require two factors for the structural integrity of their voluntary muscles. The muscular dystrophy produced on this diet has been prevented or cured by alpha-tocopherol without the addition of a water-soluble factor.

2. A quantitative relationship between alpha-tocopherol and a water-soluble factor could not be demonstrated.

3. Preventive and curative experiments place the average daily antidystrophy requirement of the rabbit for alpha-tocopherol between 0.6 and 1.0 mg. per kilogram of body weight.

4. The production of chronic dystrophy has been described and the symptoms discussed with reference to the action of vitamin E in the rabbit.

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RELATION OF PANTOTHENIC ACID TO THE FILTRATE FRACTION OF THE VITAMIN B COMPLEX

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The occurrence of an experimentally produced nutritional dermatitis in chickens was first described by Ringrose, Norris and Heuser ('31). Kline, Keenan, Elvehjem and Hart ('32) introduced the heated diet upon which dermatitis could be produced quite regularly. Elvehjem and Koehn ('35) showed this diet to be deficient in a "filtrate factor," while Lepkovsky and Jukes ('35) reported the heated diet to be also low in riboflavin. The latter investigators supplemented the diet with a fuller's earth adsorbate of riboflavin prepared from whey, thereby improving the heated diet as a test for their "filtrate factor." Bauernfeind and Norris ('39) subsequently found the heated diet to be deficient in a new growth factor which they showed could be adsorbed on fuller's earth.

The heated diet has been widely used to evaluate quantitatively the amount of chick antidermatitis vitamin ("filtrate factor") in foodstuffs. Waisman, Mickelsen and Elvehjem ('39) determined the minimum amount of foodstuff necessary to prevent dermatitis and made their comparisons on this basis. Jukes and Lepkovsky ('36) used growth over a 14-day period as the basis for their "filtrate factor unit." The use of growth measurements for quantitative comparisons would be valid only if there were no other growth limiting deficiency in the diet, but the findings of Bauernfeind and Norris ('39) indicate the heated diet may not meet this requirement.

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The chick antidermatitis vitamin has been shown by Woolley, Waisman and Elvehjem ('39) and Jukes ('39) to be similar to pantothenic acid. Filtrates containing the chick antidermatitis vitamin also contain a factor or factors necessary for the rat, and such filtrates have been studied with rats to secure information on this factor (factor 2 or "rat filtrate factor"). Hitchings and Subbarow ('39) on the basis of stability data and Macrae, Todd, Lythgoe, Work, Hind and El Sadr ('39) on the basis of solubility have claimed that the "filtrate factor" consists of two or more growth-promoting vitamins. On the basis of solubility in ether from strongly acidified solutions, Mohammad, Emerson, Emerson and Evans ('40) and Kringstad and Lunde ('39) have concluded that the chick antidermatitis vitamin is different from the "rat filtrate factor."

Very potent concentrates of pantothenic acid were shown by Oleson, Woolley and Elvehjem ('39) and Subbarow and Hitchings ('39) to stimulate growth in rats. Woolley ('40) confirmed this with pure pantothenic acid and was able to show definitely that rats require an unidentified factor of the vitamin B complex or an unidentified water-soluble factor in addition to pantothenic acid, thiamin, riboflavin and pyridoxin.

Lunde and Kringstad ('39) and Morgan and Simms ('40) have reported the occurrence of an "anti-grey hair factor" in "filtrates" which appears to be different from the known vitamins. Gyorgy, Poling and Subbarow ('40) have found pantothenic acid to play an important role in the cure of grey hair. Their pantothenic acid concentrates were about 50% pure. Oleson, Elvehjem and Hart ('39) have shown that while grey hair is not prevented with pantothenic acid, it does definitely delay the onset of the symptoms.

Nielsen, Oleson and Elvehjem ('40) have described the procedure for the preparation of a crystalline fraction from liver, which is not pantothenic acid, but which seems to be specifically active in the prevention of the greying of black rats' hair (nutritional achromotrichia). It is possible that nutritional achromotrichia is influenced by several factors.

The synthesis of pantothenic acid by Williams and Major ('40) and Weinstock, Arnold, May and Price ('40) has made possible a further study of the "filtrate" factors of the vitamin B complex for both chickens and rats.

EXPERIMENTAL

Chicks. Day-old chicks were placed on the U. C. Poultry Mash (Lepkovsky and Jukes, '35) for 1 week and maintained on the heated diet for 1 week at which time the tests were started. The composition of the heated diet is as follows:

	%	
Whole ground corn	55.25	Heated at 120°C.
Wheat middlings	25.00	for 24 hours
Extracted casein [1]	12.00	
B-80 adsorbate [2]	2.50	
Sodium chloride	1.00	
Ground limestone	1.00	
Steamed bonemeal	1.00	
Cod liver oil	2.00	
Alfalfa leaf meal	0.25	

All chicks were allowed the diet and water ad libitum.

The test materials were diluted to a convenient volume for feeding. They were fed by inserting a pipette into the esophagus of the chick and allowing the material to flow into the crop. The doses were fed daily with a double dose on Saturday to take care of the Sunday feeding.

Development and disappearance of typical chick dermatitis were noted throughout the experimental period.

Rats. Twenty-one-day-old rats weighing 40 to 50 gm. were placed in individual cages with wire screen bottoms and fed the basal diet (Dimick and Schreffler, '39). The diet consisted in per cent of sucrose 59, extracted casein 27, primex 8, cod liver oil 2, and salt mixture no. 185 (McCollum and Simmonds, '18) 4. It was supplemented daily with 25μg. each of thiamin, riboflavin and pyridoxin.

[1] As described by Dimick and Schreffler ('39).

[2] B-80 adsorbate prepared from rice bran concentrate was assayed separately and contained 80 U.S.P. units of thiamin, 60 μg. of riboflavin and 50 μg. of pyridoxin per gram.

The rats were weighed weekly and when they failed to gain, they were started on the test materials. This occurred in from 4 to 5 weeks when the body weight was from 55 to 75 gm. The fur was ruffled, greasy in appearance and showed indications of greying. Some rats had developed reddening and swelling of the eyelids with an exudate which frequently caused the lids to stick together.

Dietary supplements. A rice bran concentrate [3] was used as the source of the vitamin B complex. Microbiological tests [4] as used by Weinstock et al. ('40) showed this concentrate to contain 400 μg. of pantothenic acid per cubic centimeter. A microbiologically assayed crude preparation of synthetic pantothenic acid served as our source of this factor.[5]

The eluate was prepared as follows: 500 gm. fuller's earth were shaken with 1 liter of rice bran concentrate diluted with 5 volumes of water at pH 5.5. This adsorbate was washed thoroughly. It was eluted with 0.1 N barium hydroxide solution. After removal of the barium the remaining liquid was concentrated under vacuum.

The rice bran filtrate fraction was prepared as follows: The rice bran concentrate was treated twice with fuller's earth using 500 gm. fuller's earth for each 1 liter of rice bran concentrate diluted to 5 volumes with water. The filtrate was concentrated and fermented with yeast to remove the sugar. It was adjusted to pH 7 with calcium hydroxide, concentrated, and methanol added to a concentration of about 80% by volume. It was then filtered and concentrated. This concentrate was adjusted to pH 2 and extracted in the cold six times with 80% methyl ethyl ketone. The methyl ethyl ketone insoluble fraction, after removal of the ketone, was treated three times with charcoal at pH 4–5. Two hundred and fifty

[3] Essentially an aqueous extract of rice bran commercially known as "Vitab Type II Liquid."

[4] We are indebted to Dr. Aaron Arnold, National Oil Products Company, for the microbiological tests of rice bran concentrate (Vitab Type II Liquid), pantothenic acid, and rice bran filtrate fraction.

[5] This was supplied by Doctors Weinstock, Arnold, May and Price, National Oil Products Company.

grams of charcoal and 1 liter of ketone insoluble fraction, diluted with 5 volumes of water, were used for each adsorption. The final filtrate, concentrated to a thick syrup, contained only 14 μg. of pantothenic acid per cubic centimeter by microbiological test.[6]

Feeding experiments. Groups of chicks were fed the rice bran concentrate at levels of 1.0, 0.5, 0.25 and 0.125 cc. respectively per chick per day. The pantothenic acid at each level was calculated and other groups of chicks were fed equivalent levels of the synthetic pantothenic acid. The differences in growth between chicks on the rice bran concentrate and those on pantothenic acid were noted, and, since these differences might well be due to the factor of Bauernfeind and Norris ('39) this factor was fed as a barium hydroxide eluate of fuller's earth. One-tenth cubic centimeter of the eluate per chick per day was given to half of each group of chicks which had previously received 400 and 200 μg. of pantothenic acid respectively for the 3 weeks' test period.

Rats were similarly studied. The rice bran concentrate containing 400 μg. pantothenic acid per cubic centimeter was fed at levels of 1.0 and 0.5 cc. respectively. Synthetic pantothenic acid was fed at levels of 800, 400 and 200 μg. respectively. Since the rice bran concentrate promoted greater growth in the rats than synthetic pantothenic acid it seemed logical to assume that the concentrate was furnishing an unidentified water-soluble factor or factors of the vitamin B complex. Accordingly, the rice filtrate fraction containing negligible amounts of pantothenic acid was fed in addition to pantothenic acid. Three groups of rats received 50, 100 and 200 μg. pantothenic acid respectively, and three groups received the corresponding amounts of pantothenic acid plus 0.5 cc. rice bran filtrate fraction. This level supplied 7 μg. of pantothenic acid which amount was subtracted in feeding the pantothenic acid doses.

[6] See footnote 4, page 416.

RESULTS

Chicks. The average growth rates of chicks fed pantothenic acid was inferior to that of chicks fed the same amount of pantothenic acid in the form of rice bran concentrate. This is shown in table 1.

TABLE 1

Comparative growth of chicks receiving rice bran concentrate and equivalent levels of pantothenic acid

SUPPLEMENT	NUMBER OF CHICKS	AVERAGE BODY WEIGHT AT				Gain	DERMATITIS
		Beginning	1 week	2 weeks	3 weeks		
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	
Rice bran concentrate 1.0 cc.: equivalent to 400 µg. pantothenic acid	8	83	127	190	237	154	None
400 µg. pantothenic acid	8	82	122	166	210	128	None
Rice bran concentrate 0.5 cc.: equivalent to 200 µg. pantothenic acid	8	83	116	186	230	147	None
200 µg. pantothenic acid	8	84	115	171	203	119	None
Rice bran concentrate 0.25 cc.: equivalent to 100 µg. pantothenic acid	8	83	109	152	190	107	4 mild cases
100 µg. pantothenic acid	8	83	106	125	161	78	6 severe cases
Rice bran concentrate 0.125 cc.: equivalent to 50 µg. pantothenic acid	8	83	100	134	149	66	2 died 3 severe 2 mild
50 µg. pantothenic acid	8	83	92	127	144	61	3 died 4 severe 1 mild

The chicks receiving 1.0 and 0.5 cc. levels of rice bran concentrate respectively, as well as those receiving 400 and 200 μg. levels of pantothenic acid respectively, were completely free of dermatitis. Those receiving the next lower levels of rice bran concentrate and pantothenic acid showed incomplete protection, while the lowest levels afforded the chicks only slight protection. The protection against chick dermatitis seemed consistent with the level of pantothenic acid fed whether from rice bran concentrate or as the synthetic preparation.

The difference in growth between chicks receiving the rice bran concentrate and those given synthetic pantothenic acid

TABLE 2

Growth of chicks receiving pantothenic acid with and without rice bran eluate

SUPPLEMENT	NUMBER OF CHICKS	AVERAGE BODY WEIGHT AT Beginning	1 week	2 weeks	Gain
		gm.	*gm.*	*gm.*	*gm.*
200 μg. pantothenic acid	4	217	249	286	69
200 μg. pantothenic acid plus 0.1 cc. eluate	4	202	255	328	116
400 μg. pantothenic acid	4	213	269	308	95
400 μg. pantothenic acid plus 0.1 cc. eluate	4	208	271	338	130

indicated that another factor is necessary for the chick fed the heated diet supplemented with pantothenic acid. The lack of this factor was not evident until after the end of the first week of the test period, but became striking by the end of 3 weeks. On this basis, it would seem that dermatitis, used by Waisman, Mickelsen and Elvehjem ('39), may be a better criterion than growth, used by Jukes and Lepkovsky ('36), in the quantitative assay of foodstuffs for pantothenic acid.

The results of feeding an eluate in addition to 400 and 200 μg. pantothenic acid are shown in table 2. The eluate increased the growth in both cases, showing that it must contain a factor other than pantothenic acid. As the factor can be

adsorbed on fuller's earth, it may be similar to that of Bauernfeind and Norris ('39), who have shown that the mature fowl, when fed the heated diet supplemented with pantothenic acid, supplied by rice bran filtrate, also requires an adsorbable factor. Our factor may also be related to factor U of Stokstad and Manning ('38) which has similar properties.

Rats. The results with rats are shown in tables 3 and 4. The rice bran concentrate supported a better rate of growth than synthetic pantothenic acid (table 3), even when four times as much pantothenic acid as that supplied by the rice

TABLE 3

Comparative growth of rats receiving rice bran concentrate and panthothenic acid

SUPPLEMENT	NUMBER OF RATS	SEX	AVERAGE BODY WEIGHT AT Beginning	7 weeks	Gain
			gm.	*gm.*	*gm.*
Rice bran concentrate 0.5 cc.: equivalent to 200 μg. pantothenic acid	6	Females	60	191	131
200 μg. pantothenic acid	6	Females	67	172	105
Rice bran concentrate 1.0 cc.: equivalent to 400 μg. pantothenic acid	4	2 males 2 females	71	233	162
400 μg. pantothenic acid	3	2 males 1 female	72	154	82
800 μg. pantothenic acid	3	2 males 1 female	99	196	97

bran concentrate was fed. Pantothenic acid cured the eye symptoms, improved the fur condition and stimulated rapid growth. The growth of rats receiving synthetic pantothenic acid, however, was not as marked as that of rats receiving equivalent amounts of pantothenic acid present in the rice bran concentrate. Rats which were grey on the back, as well as one which had the fur missing in patches on the sides with slight skin eruptions exhibited growth of fur of a normal color, and the skin eruptions cleared up when pantothenic acid was fed. However, the new fur was subsequently inclined to

turn grey and considerable greying persisted. Twelve rats (used for other experiments) were fed 200 μg. daily of synthetic pantothenic acid from the day of weaning in addition to the same basal diet and 25 μg. each of thiamin, riboflavin, and pyridoxin. At 3 months of age, nine of the twelve showed

TABLE 4

Comparative effect of feeding various levels of pantothenic acid with and without the rice bran filtrate fraction with adjustment for the pantothenic acid contained in the filtrate fraction

SUPPLEMENT	NUMBER OF RATS	AVERAGE BODY WEIGHT AT Beginning	7 weeks	Gain	FUR CONDITION
	Females	*gm.*	*gm.*	*gm.*	
50 μg. pantothenic acid	4	58	111	53	Grey
43 μg. pantothenic acid plus 0.5 cc. filtrate fraction containing 7 μg. pantothenic acid	5	59	150	91	3 grey 2 restored
100 μg. pantothenic acid	4	57	147	90	grey
93 μg. pantothenic acid plus 0.5 cc. filtrate fraction containing 7 μg. pantothenic acid	4	57	167	110	3 restored 1 some grey remaining
200 μg. pantothenic acid	5	63	150	87	Some grey
193 μg. pantothenic acid plus 0.5 cc. filtrate fraction containing 7 μg. pantothenic acid	4	59	177	118	None grey

slight signs of greying. This indicates that pantothenic acid does not completely prevent greying.

The rats receiving the rice bran concentrate were superior in that respect. This is in agreement with previous unpublished experiments with rice bran concentrates which have been shown to contain a factor or factors which will prevent greying and restore the normal fur color in rats which have become grey.

This is in agreement with the published results of Morgan and Simms ('40), which have shown rice bran concentrate to be an excellent source of the "anti-grey hair factor."

The data in table 4 point definitely to an unidentified "filtrate factor" which escapes, at least in part, adsorption by fuller's earth and charcoal. Rats receiving the rice bran filtrate fraction in addition to pantothenic acid grew better at all levels than those on the corresponding levels of pantothenic acid. The hair color of rats receiving 200 μg. pantothenic acid plus the filtrate fraction was completely restored in the 7 weeks' test period. The hair of the rats receiving the 100 μg. pantothenic acid plus the filtrate fraction was definitely black with the greying disappearing rapidly. This was in contrast to those on pantothenic acid alone and similar to those on the rice bran concentrate. When fed alone, the rice bran filtrate fraction possessed no demonstrable biological activity.

DISCUSSION

It is clear that the vitamin B complex consists of still unidentified additional factors. Evidence has been presented to show that at least two unidentified factors belonging to the vitamin B complex are present in a concentrate prepared from rice bran. These factors would appear to be different since one, needed by the chick, is adsorbable on fuller's earth; and the other, needed by the rat, is found in filtrates after treatment with fuller's earth and charcoal. Bauernfeind and Norris ('39) showed adsorbates to be effective in the chick in the presence of rice bran filtrate.

Pantothenic acid is an important fraction of the rat "filtrate factor" or factor 2, but does not constitute all of it by any means. It supports rapid growth, but at a rate lower than that obtained when a rice bran concentrate is fed. Pantothenic acid clears up such minor factor 2 deficiency symptoms as swollen eyelids and occasional skin eruptions. The rats have a slightly greasy appearance with ruffled fur, and grey hairs sprinkled throughout, but are otherwise not very different from normal animals.

Pantothenic acid seems to be an important component of the "anti-grey hair factor," delaying greying and causing darkening of the fur already grey. Nielsen, Oleson and Elvehjem ('40) have prevented greying with a crystalline preparation from liver. The relation between this factor and pantothenic acid in the prevention of greying is, at present, not clear. Some vitamins such as pyridoxin and nicotinic acid (Morgan and Simms, '40; Lunde and Kringstad, '40) accelerate the greying of the rats' fur. It would seem that greying is a phenomenon involving many factors whose interrelationships are at present not well understood.

Our results show that, in the chicken, pantothenic acid is definitely identical with the chick antidermatitis vitamin, but only insofar as prevention and cure of dermatitis is concerned. It does not comprise all of the filtrate factor, since the filtrate factor is measured in terms of growth on the heated diet and since it has been demonstrated that there is at least one unidentified growth factor other than pantothenic acid which is inadequately supplied by the heated diet.

The heated diet is satisfactory for the quantitative estimation of pantothenic acid when the prevention of dermatitis is the criterion used (Waisman, Mickelsen and Elvehjem, '39). If growth is the criterion, however, only those foodstuffs free of unidentified growth promoting factors will give reliable results.

CONCLUSIONS

1. Synthetic pantothenic acid will cure or prevent an experimentally produced nutritional dermatitis which develops in chicks on a diet of natural foodstuffs heated at 120°C. for 24 hours. It does not appear to represent the whole of the factor referred to as the "filtrate factor" since it produces a growth response below that obtained with rice bran concentrate.

2. An eluate prepared from rice bran concentrate contains a factor necessary for the growth of chicks on the heated diet

of natural foodstuffs in addition to pantothenic acid. This confirms the findings of Bauernfeind and Norris ('39).

3. Pantothenic acid is an important part of factor 2 ("rat filtrate factor"). It promotes growth of rats, cures the reddening and swelling of the eyelids which develop in rats on a factor 2-deficient diet, improves the skin condition and decreases greying of the fur; but it does not completely restore hair color nor does it completely prevent greying.

4. From a rice bran concentrate, a filtrate fraction low in pantothenic acid has been prepared which is shown to contain an unidentified factor necessary for the rat.

5. Rice bran concentrate contains a chick and a rat growth factor (or factors) in addition to the now recognized components of the vitamin B complex, namely, thiamin, riboflavin, pyridoxin, nicotinic acid and pantothenic acid.

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VITAMIN C METABOLISM IN EASTERN COTTON RATS [1]

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Recent demonstration of the susceptibility of the eastern cotton rat to the virus of poliomyelitis (Armstrong, '39; Jungeblut and Sanders, '40) prompted a systematic investigation in this laboratory of the response of this animal to other infectious and toxic agents. The significant finding which emerged from these comparative studies was the fact that the cotton rat in its reaction to diphtheria toxin (Jungeblut, '40), histamine poisoning (Seegal, '40) and experimental tuberculosis (Steinbach and Duca, '40) followed a rather uniform pattern. To be more precise, its tolerance to these agents seemed to correspond with that of an animal occupying a position, within the rodent group, midway between the extremely susceptible guinea pig, on the one hand, and the extremely insusceptible albino rat, on the other. Admittedly, the mechanism involved in the various manifestations of natural tolerance is still far from being well understood; however, there are reasons for believing that basic differences in vitamin C metabolism may play an important, if not decisive part, at least in the above-mentioned instances (Perla and Marmorston, '37).

In view of the described peculiar biological grouping of the cotton rat it has seemed of interest to determine whether this animal, with respect to its vitamin C metabolism, should

[1] Supported by a grant from the Philip Hanson Hiss, Jr., Memorial Fund.

be regarded as belonging to the non-synthesizing type of the guinea pig, or to the synthesizing type of the albino rat. Ascorbic acid determinations were therefore carried out in which the vitamin C levels in the tissues of cotton rats were compared with those of guinea pigs and albino rats. In further experiments, the effect of withdrawal of vitamin C from the diet upon the vitamin C content of representative organs was studied in the same three species.

Florida cotton rats (Sigmodon hispidus littoralis), weighing between 100 and 175 gm., were used in this work. Before being placed on an experiment, the animals had been kept in the laboratory for at least 30 days on an adequate diet (oatmeal, raisins, apples and greens). The albino rats were laboratory-bred (weight from 100 to 150 gm.) and had been maintained on a modified McCollum diet. The guinea pigs (weight from 250 to 300 gm.) had been on an adequate laboratory diet (oats, hay and greens) for short periods of time, ranging from 4 to 10 days. All animals were sacrificed and the tests made on fresh tissues. The organs examined for vitamin C content were liver, brain, spleen and adrenal. The modified method of Bessey and King ('33) and Musulin and King ('36) was employed for determining the amounts of reduced ascorbic acid present in these tissues.

Vitamin C content of tissues of cotton rats, albino rats and guinea pigs

During the spring and early summer months, a total of eight cotton rats were killed and ascorbic acid titrations made on representative tissues. On the same days, for purpose of comparison, determinations were also made on the tissues of albino rats and guinea pigs. The results are given in table 1.

It will be seen that the average figures for the vitamin C content of liver, brain and spleen show no significant differences between cotton rats and albino rats. However, the vitamin C levels in the adrenals of cotton rats were definitely lower than those in the adrenals of albino rats. Thus, the

TABLE 1

Vitamin C content of the tissues of cotton rats and effect of scorbutic diet on its level as compared with the effect on the level in tissues of albino rats and guinea pigs

ANIMAL	NUMBER	DIET	NUMBER OF DAYS ON DIET	MILLIGRAMS OF ASCORBIC ACID PER GRAM OF FRESH TISSUE			
				Liver	Brain	Spleen	Adrenals
Guinea pig	10 (average)	Stock	4–10	0.075	0.085	0.082	0.29
Guinea pig	2	Scorbutic + ascorbic acid	15	0.13	0.14	0.12	0.501
Guinea pig	1	Scorbutic + ascorbic acid	22	0.089	0.15	0.19	0.38
Guinea pig	1	Scorbutic + ascorbic acid	30	0.11	0.12	0.23	0.36
Guinea pig	1	Scorbutic + ascorbic acid	31	0.15	0.15	0.21	0.55
Guinea pig	2	Scorbutic	15	0.018	0.017	0.019	0.036
Guinea pig	1	Scorbutic	22	0.035	0.017	0.02	0.044
Cotton rat	8 (average)	Stock	+30	0.22	0.28	0.34	1.13
Cotton rat	2	Scorbutic + ascorbic acid	17	0.37	0.32	0.37	1.14
Cotton rat	1	Scorbutic + ascorbic acid	25	0.22	0.30	0.58	1.10
Cotton rat	1	Scorbutic + ascorbic acid	37	0.25	0.29	0.50	1.09
Cotton rat	1	Scorbutic + ascorbic acid	51	0.28	0.33	0.46	1.32
Cotton rat	2	Scorbutic	17	0.31	0.29	0.44	0.934
Cotton rat	1	Scorbutic	25	0.22	0.32	0.40	0.99
Cotton rat	1	Scorbutic	37	0.27	0.28	0.47	0.85
Cotton rat	1	Scorbutic	51	0.29	0.30	0.54	1.27
Albino rats	16 (average)	Stock	±60	0.23	0.32	0.28	2.59
Albino rat	2	Scorbutic + ascorbic acid	18	0.18	0.31	0.23	2.70
Albino rat	1	Scorbutic + ascorbic acid	25	0.15	0.35	0.21	3.21
Albino rat	1	Scorbutic + ascorbic acid	39	0.21	0.31	0.25	3.46
Albino rat	1	Scorbutic + ascorbic acid	50	0.25	0.33	0.34	2.95
Albino rat	1	Scorbutic + ascorbic acid	51	0.24	0.31	0.29	3.05
Albino rat	2	Scorbutic	18	0.11	0.20	0.15	2.67
Albino rat	1	Scorbutic	25	0.21	0.34	0.23	3.32
Albino rat	1	Scorbutic	39	0.20	0.31	0.28	3.33
Albino rat	1	Scorbutic	50	0.20	0.31	0.29	2.93
Albino rat	1	Scorbutic	51	0.17	0.32	0.30	3.39

average vitamin C content, expressed in milligrams of ascorbic acid per gram of fresh tissue, was 2.59 in the adrenals of the albino rat (range 2.00 to 3.34), whereas in the cotton rat it was only 1.13 (range 1.10 to 1.15). On the other hand, the average vitamin C levels of all cotton rat tissues were considerably higher than those of the corresponding tissues for guinea pigs. Although the average figures for guinea pigs given in this table are clearly below normal, probably due to a combination of inadequate diet and seasonal changes (Jungeblut and Feiner, '39), yet the values obtained for the tissues of other guinea pigs, known to have received ample amounts of ascorbic acid, remained consistently below those of cotton rat tissues.

The effect of withdrawal of vitamin C from the diet on the vitamin C content of the tissues of cotton rats, albino rats and guinea pigs

A total of twelve cotton rats, twelve albino rats and twelve guinea pigs were included in this experiment. After having been kept on a preliminary stock diet for 7 days, the members of each species were distributed into two groups of six animals each. One group was fed a modified Sherman scorbutic diet, consisting of rolled oats, skimmed milk powder, cottonseed oil,[2] cod liver oil and sodium chloride, with wheat bran for the guinea pigs and ground whole wheat for the rats. The other group was maintained on the same scorbutic diet but received in addition large doses of synthetic ascorbic acid[3] dissolved in fresh orange juice. Each animal ingested daily approximately from 5 to 10 mg., an amount many times the prophylactic antiscorbutic dose for guinea pigs. At intervals, animals were killed and ascorbic acid determinations made on representative tissues from animals maintained on the scorbutic diet, both with or without supplementary ascorbic acid. The results are shown in table 1. The data given for guinea pigs and cotton rats are incomplete because of intercurrent deaths in the two groups of animals.

[2] Wesson.

[3] Hoffman-LaRoche.

It appears from this table that cotton rats, like albino rats, on an ascorbic acid-free diet from 17 to 51 days, were able to maintain normal vitamin C levels in their tissues, approximating those attained with the same diet supplemented by an additional intake of ascorbic acid. The slight drop observed in the adrenal of cotton rats fed the scorbutic diet is too small to be statistically significant. Moreover, none of the rats showed any symptoms of scurvy and all remained in good health throughout the entire course of the experiment. Data for only three guinea pigs receiving the scorbutic diet without supplementary ascorbic acid are available as the remainder of this group died of intercurrent infections. As might have been expected, these figures revealed a marked drop in the vitamin C levels of all tissues examined; this drop was more than compensated for by the supplementary intake of ascorbic acid in the protected parallel group.

SUMMARY AND CONCLUSIONS

The results reported in this paper show that the ascorbic acid content of the liver, spleen and brain of the cotton rat stands at about the same level as that of corresponding tissues of the albino rat. These concentrations are, of course, considerably above those of the guinea pig. However, the vitamin C level of the adrenal in cotton rats is markedly below that in albino rats and begins to approach the figures for guinea pigs. In spite of this relatively low adrenal storage, the cotton rat seems perfectly capable of maintaining, throughout its tissues, a normal threshold of vitamin C when deprived of ascorbic acid in its diet. Moreover, an additional intake of ascorbic acid is apparently not utilized since it fails to raise the vitamin C values beyond their existing limits for each organ. This would suggest that the cotton rat, like the albino rat, possesses some physiological mechanism for synthesizing vitamin C, in sharp contrast to the demonstrated inability of the guinea pig in this respect. The vitamin C metabolism of cotton rats may therefore be said to compare essentially with that of albino rats, save for the relatively

low adrenal storage of ascorbic acid, which resembles somewhat the characteristics of the guinea pig.

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A COMPARISON OF THE NUTRITIVE VALUES OF RAW, PASTEURIZED AND EVAPORATED MILKS FOR THE DOG [1]

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THREE TEXT FIGURES AND ONE PLATE (SIX FIGURES)

(Received for publication May 31, 1940)

The wide use of commercially processed milks in infant nutrition has raised several questions concerning the nutritive value of these milks in comparison with the original raw milk. It is still impossible to compare on a quantitative basis all the nutrients present in the various milks because assay procedures are not sufficiently perfected. Elvehjem, Hart, Jackson and Weckel ('34) compared the nutritive value of raw and pasteurized milks by following the growth of rats on these milks after mineralization with iron, copper, and manganese. When the experiments were conducted for only 6 weeks, greater differences were observed between winter and summer produced milks than between raw and pasteurized milks. Other animals, including swine (Kemmerer et al., '32) and dogs (Potter et al., '38), have been maintained for long periods of time on mineralized raw milks. Recently Evans and Phillips ('39) raised rats through five generations on milk supplemented with iron, copper, and manganese.

We felt that if differences did exist between various milks it might be possible to demonstrate such differences through

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from the University Research Fund and the Works Progress Administration.

reproduction experiments with dogs. The results reported in this paper must be considered preliminary in nature both because of the limited number of dogs used and because no attempt was made to control the source of the milk used for the processed products. Nevertheless the requirements of dogs are evidently sufficiently divergent from those of rats to demonstrate certain differences in the various milks.

EXPERIMENTAL

The original experiment was set up with one male and one female dog on each of the following milks: raw, obtained from several cows in the university herd maintained on a standard ration [2] throughout the experiment, pasteurized and evaporated (non-irradiated). The animals were fox terrier litter mates and were started on the milk diets when 9 weeks of age. They were kept in galvanized wire pens on a dry concrete floor covered with shavings. The daily mineral supplement per dog included 5 mg. of Fe as the pyrophosphate, 1 mg. Cu as the sulfate and 1 mg. Mn as the chloride and was added to a small amount of milk for the morning feeding to insure complete consumption. The evening feeding of milk was large enough to allow ad libitum consumption. Cod liver oil was added to the ration of all the dogs at the points designated on the growth curves.

The dogs were weighed weekly and the following blood determinations were made periodically: calcium, inorganic phosphorus, hemoglobin, cell volume, and cell count. No significant differences were observed between the groups. Data for calcium and phosphorus on the individual dogs have been published separately (Anderson and Elvehjem, '40). The young obtained from the original pairs of dogs were used as additional animals on the various milks.

Raw milk. The growth curves for the original pair of dogs on raw milk are given in figure 1. Grossly there has never

[2] The winter ration consisted of corn silage, timothy hay, and a grain mixture of corn and oats. For some of the cows part of the grain mixture was replaced by urea or linseed oil meal as a nitrogen source. Milk from these cows was consistently low in the grass juice factor.

been any indication that these dogs were suffering from a nutritional deficiency (figs. 1 and 2, pl. 1). However, the response in blood calcium and inorganic phosphorus when cod liver oil was administered (Anderson and Elvehjem, '40) indicates that there was a borderline deficiency of vitamin D. From point A in figure 1 the dogs were given 30 International units of vitamin D per day and at point B the level was increased to 125 I.U. per day.

At the point "D" dog 5 gave birth to five pups. Of these, two died within 18 hours, and another the following week. One of the pups was accidentally killed by the dam about the time of weaning. The remaining pup grew rather slowly in com-

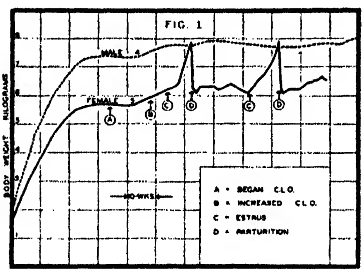

Fig. 1 Growth curves for dogs on raw milk, beginning the ninth week post partum. Dog 5 had litters of five pups each time.

parison to the original litter but otherwise showed no gross evidence of a nutritional deficiency. This pup was maintained on the raw milk ration with added cod liver oil and minerals for a year, and was then transferred to another experiment.

At the next point "D" dog 5 gave birth to a litter of five pups, one of which died at birth. The others, apparently vigorous pups, grew well showing no signs of a nutritional deficiency.

In a previous report (Anderson et al., '39) we described a deficiency in dogs on the evaporated milk ration which responded to vitamin E therapy. To ascertain whether or not the young dogs on the raw milk were deficient in this factor, two pups from this litter were given 5 mg. of dl-α-tocopherol-

acetate [3] per dog per week. The other two dogs were used as controls. The growth of the animals in the two groups was very similar and no dystrophy [4] was evident. Two of these dogs have been continued on the raw milk diet and have been bred in order to determine if the third generation can be reared successfully.

Evaporated milk. Figure 2 gives the data on the original pair of dogs maintained on the mineralized evaporated milk plus cod liver oil. The male (dog 2) showed no indication of nutritional deficiency (fig. 3, pl. 1) until the dog has been on experiment for 2 years. At this time (point C on curve) this

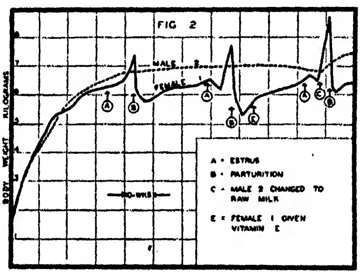

Fig. 2 Growth curves for dogs on evaporated milk, beginning the ninth week post partum. Dog 1 had litters of three, five and six pups respectively. Note the increase in body weight of dog 2 when changed to a diet of raw milk.

dog was transferred to raw milk and showed an immediate increase in body weight. Since this increased weight has been maintained for a period of 5 months and the level is significantly higher than the weight level he maintained on the evaporated milk, it would appear that the evaporated milk was not quite optimal for growth and maintenance of the male dog.

[3] We are indebted to Hoffmann La Roche, Inc., Nutley, New Jersey, for generous supplies of synthetic dl-α-tocopherol-acetate.

[4] The term muscle dystrophy is used in this paper to designate the general muscular weakness. Since we have treated most of the dogs showing any muscular weakness with vitamin E, we do not have sufficient material for extensive histological studies.

The female (dog 1) has had three litters of pups to date. The muscle dystrophy occurring in the pups of the first two litters has already been described (Anderson et al., '39). Two of the dogs in the second litter were protected from dystrophy by the administration of vitamin E (figs. 4 and 5, pl. 1). The condition of dog 1 during the second period of lactation was also described; and due to the severe skin lesions on the dam and the rapid loss of weight, the young were placed on mineralized evaporated milk on the eighteenth day of age. Definite muscle dystrophy did not develop in these dogs but the growth response obtained when dl-α-tocopherol was added indicated that vitamin E was still a limiting factor.

In order to determine if a completely normal litter could be produced by giving vitamin E during pregnancy, dog 1 was given 10 mg. of the tocopherol acetate per week beginning at point E and increased to 5 mg. per day during gestation. A very striking difference was observed between this gestation period and the former one with regard to the weight increase. In previous gestations the animal's weight had not increased in the last 5 days before parturition and in both cases she had lost weight during the last 2 days before parturition. In this gestation, when she received the added vitamin E, her weight continued to increase until the day of parturition. She also gained a kilogram more in weight than in the previous gestation yet gave birth to only one more pup.

The pups were somewhat larger than those of former litters yet all died within 3 days. Externally the pups showed a considerable number of lesions on the legs, joints, lower jaw and abdomen. The lesions varied in diameter from 1 to 8 mm. Some had formed scabs; others were apparently open lesions. From gross examination these lesions were quite similar to those which developed on the dam in the previous lactation period. Since the pups did not survive, it is impossible to determine if the added vitamin E would have given protection against the muscle dystrophy.

Autopsies on the pups showed considerable amounts of free blood in the abdominal cavity. Similarly hemorrhagic areas

were observed in the pericardium, lungs, and subcutaneous areas. In many cases these hemorrhages were of such severity that they gave a decided blue black color to the area as early as 24 hours before the death of the animal. Perhaps the most striking observation was the fact that the intracranial blood vessels were severely engorged. In three cases they had ruptured and severe hemorrhages had resulted.

Bacteriological examinations of the organs all showed negative results, and histological studies confirmed these observations since there was no apparent infection but rather a generalized congestion and extensive hemorrhage of the blood vessels.

Pasteurized milk. Because results indicated that the pasteurized milk had a nutritive value between that of the raw and the evaporated milks, photographs and growth curves will not be included in this report.

In the first litter of pups born to dog 3 on pasteurized milk there was no indication of a nutritional deficiency. In the second litter, however, of the three pups born, two died within a week. Both showed the hemorrhagic condition which has been described above for the pups on evaporated milk. It would appear then that evaporated and pasteurized milks are somewhat alike in this respect.

The only pup to survive in the second litter of dog 3 was nourished entirely by the dam up to the time of weaning. At this time the pup showed many of the early signs of muscle dystrophy. Most striking was the lack of muscle tonus in the feet. Between the femur and tibia the weakness was not so striking as that in the pups on the evaporated milk but the atony of the feet was the most severe case which we have thus far encountered.

When this pup was weaned and transferred to mineralized pasteurized milk the condition improved. Hence it would appear that the pasteurized milk contains enough vitamin E to prevent muscle dystrophy in the growing dog although not an adequate amount for pregnancy and lactation.

Food consumption. Figure 3 summarizes the results of the food consumption records for the dogs. No differences occurred between the groups on raw, pasteurized and evaporated milks which might be attributed to the nutritive value of the milk. A closer correlation seemed to exist between the amount of milk consumed and the relative activity of the animal. The least active dog required 17.7 cc. of milk per gram increase of body weight, while the most active dog required 21.2 cc. Average values for the group showed that for the first 12 weeks the dogs required approximately 20 cc. of milk per gram increase of body weight. This amounts to 2.5 gm. of milk solids per gram increase of body weight.

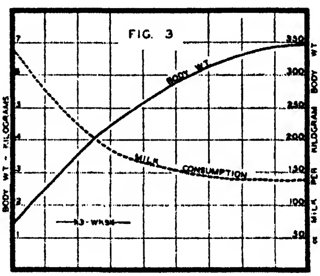

Fig. 3 Curves showing the amount of milk consumed per kilogram of body weight during the period of rapid growth.

DISCUSSION

It is apparent that whole raw milk supplemented with iron, copper, manganese, and cod liver oil will allow normal growth and reproduction in dogs. However, when pasteurized or evaporated milks are used there seems to be a limiting intake of at least two factors. One factor is related to the prevention of muscle dystrophy since all pups born to the female on evaporated milk showed definite dystrophy if they lived until 20 days of age. Since vitamin E produced beneficial effects when therapy was started early enough, the factor concerned must either be vitamin E or closely associated with it. The results indicate that pasteurized milk carries more of this factor than the evaporated milk.

It is interesting that the hemorrhagic condition did not develop in the pups until the production of the third litter in the case of the dog on evaporated milk and the second litter in the case of the animal receiving pasteurized milk. There is some possibility that the hemorrhagic condition may be related to vitamin K. The vitamin K content of the various milks is now being studied.

Herman ('36) has described a hemorrhagic condition in the kidneys, lungs, thymus, and heart of calves after 12 to 15 months on mineralized milk. Since he also used mineralized milk supplemented with cod liver oil, the same dietary factor may be involved. The dog may have a smaller requirement since no hemorrhagic condition was noted in the pups from the dog on raw milk.

All of the animals in our colony have had very excellent hair coats. Only under the strain of lactation with the dog on the evaporated milk and her dystrophic pup were there any exceptions to this condition. The excellent condition of the fur may be related to the high fat content of the milk when expressed on the dry basis.

It is impossible to apply the results obtained in this study directly in infant nutrition. Infants are rarely restricted entirely to milk diets and certainly the period of milk feeding is not comparable to the length of time that these experiments were conducted. However, the results do show that repeated pregnancy and lactation periods may bring out nutritional deficiencies which cannot be demonstrated by growth experiments alone. It should also be emphasized that the same type of pasteurized and evaporated milks were used throughout the experiment and that the results obtained with these milks may not be true for all pasteurized and evaporated milks.

SUMMARY

1. Whole raw milk supplemented with iron, copper, and manganese, and cod liver oil, allowed good growth and reproduction (two gestations) in fox terrier dogs. It was necessary to add cod liver oil in order to maintain a normal blood calcium and phosphorus level.

2. When evaporated milk was fed under similar conditions most of the young born showed either muscle dystrophy or a hemorrhagic condition of the pericardium, lungs, and brain. Vitamin E was effective in curing or preventing the muscle dystrophy but did not prevent the hemorrhagic condition.

3. The young born to the female on fortified pasteurized milk showed some signs of dystrophy and hemorrhage, but the symptoms were not as severe as those observed in the case of evaporated milk.

4. These deficiencies were only produced by repeated pregnancy and lactation since all the milks supported good growth of the males for almost 3 years and maintained the females in good condition except under the added strain of reproduction.

ACKNOWLEDGMENT

We are greatly indebted to Mr. J. J. Tamulis for valuable assistance in the care of the animals and to Mr. James H. Shaw for the histological studies.

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PLATE 1

EXPLANATION OF FIGURES

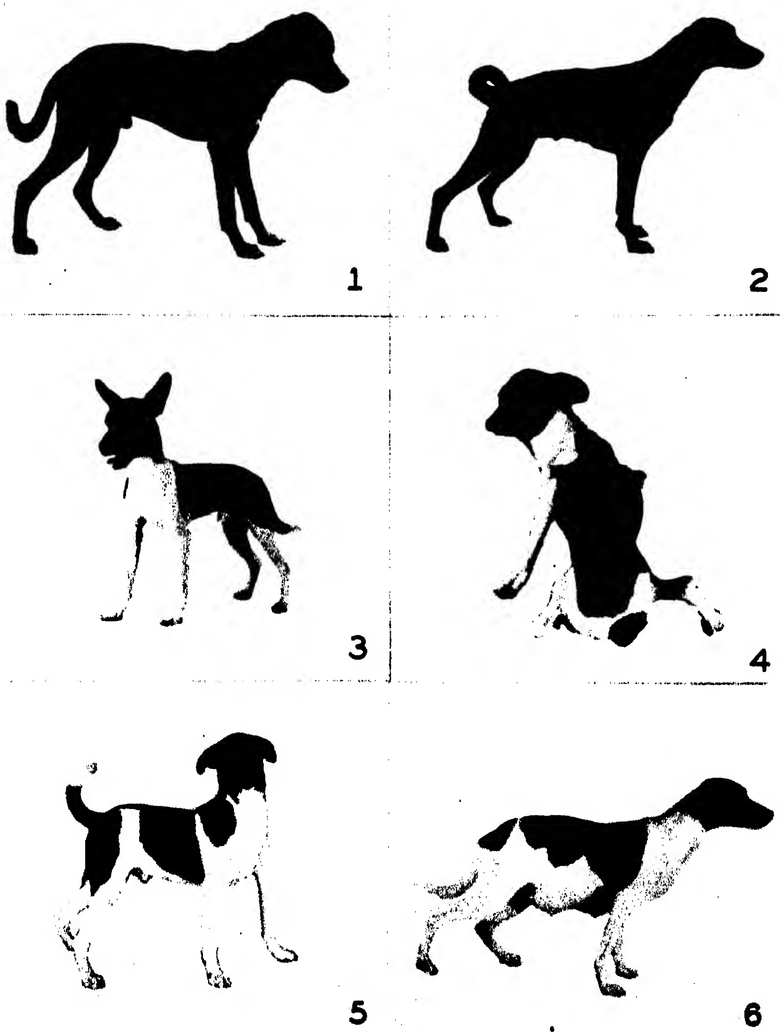
1 and 2 Dogs 4 and 5 raised on raw milk, normal after 2½ years.

3 Dog 2, raised on evaporated milk for 2½ years.

4 Dog from first litter produced by dog 1 (on evaporated milk). Pup was maintained on the fortified evaporated milk after weaning. Photograph taken when dog was 16 months old.

5 Dog from second litter produced by dog 1 (on evaporated milk). Pup was maintained on the fortified evaporated milk after weaning but 5 mg. of dl-α-tocopherol-acetate was added per week. Photograph taken when dog was 7 months old.

6 Dog 1 after the third gestation period in which she received 5 mg. vitamin E per day. Since the pups died, she was not subjected to the strain of lactation.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EFFECT OF CHOLINE AND OTHER SUPPLEMENTS ON PEROSIS

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TWO FIGURES

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Perosis, or "slipped tendon," has been extensively studied with chicks. The work, which includes many studies of the preventive effect of manganese, has been reviewed (Jukes, '37; Wilgus et al., '39). Recently it was observed (Jukes, '39 a) that turkeys were highly susceptible to perosis, and that additions of manganous sulfate to the experimental diet up to a level corresponding to 1440 parts of manganese per million of diet slightly accelerated the onset of this nutritional deformity. Injections of manganese were also ineffective. Later it was reported (Jukes, '40 a) that choline was effective in preventing perosis in turkeys.

Ringrose, Martin and Insko ('39) reported a lowering of the incidence of perosis in turkeys by the addition of manganese to an undescribed diet. Complete protection was not obtained.

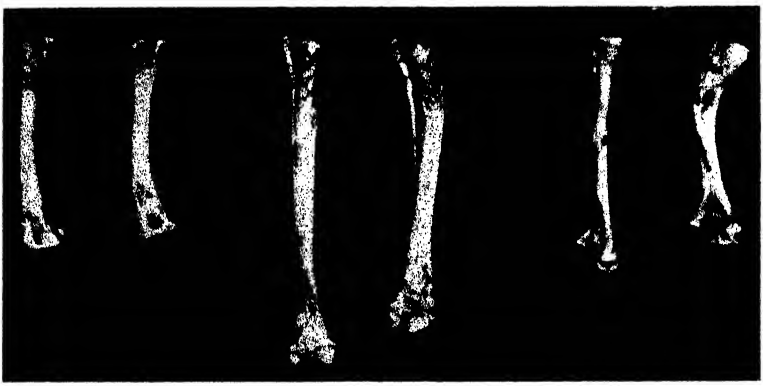
EXPERIMENTAL

Turkeys were placed in electrically heated battery brooders at hatching and were fed the experimental diets immediately. From ten to fifteen birds were used in each group. Examination for perosis was made as previously described (Jukes, '40 a).

The appearance of affected turkeys corresponded to the symptoms of perosis in chicks as outlined by the studies of

Wilgus et al. ('37), Gallup and Norris ('38), and Caskey et al. ('39). Figure 1 illustrates the leg bones from perotic and non-perotic turkeys, and is strikingly similar to figure 1 of Caskey et al. ('39).

In a preliminary series of experiments, the diet formerly employed (Jukes, '39 a) was used. It consisted of the following in percentage composition: yellow cornmeal, 63.5; dried skim milk, 15; washed casein, 13; alfalfa meal, 1; $CaCO_3$, 4; KH_2PO_4, 2; NaCl, iodized, 0.5; and fish oil blend (3000-A,



a b c d e f

Femora Tibiae Metatarsi

Fig. 1 Leg bones (a, c, e) from a turkey receiving a supplement of a synthetic mixture containing choline compared with bones (b, d, f) from a turkey on basal diet 161. The former bird weighed 207 gm., and the latter, 205 gm. when they were killed at 4 weeks of age.

400-D), 1. The following five modifications of the diet were all found to be completely ineffective in preventing perosis: (1) Substitution of limestone and bonemeal for $CaCO_3$ and KH_2PO_4 respectively; (2) replacement of 25 parts of yellow cornmeal by wheat bran; (3) addition of 2 parts of wheat bran ash to 100 parts of diet; (4) addition of 0.5 part of mineral mixture (consisting of ZnO, 5; $Al_2(SO_4)_3$, 5; $CoCl_2$, $2H_2O$, 1; ferric citrate, 89) to 100 parts of diet; (5) omission of KH_2PO_4 and halving the $CaCO_3$ content.

In succeeding experiments the diet above was modified slightly to contain 65 parts of yellow cornmeal, 3 parts of $CaCO_3$, 1 part of KH_2PO_4 and 0.1 part of $MnSO_4$ (diet 161 (Jukes, '39 a)). The other ingredients were unchanged. In each series a "positive control group" was included on a normal diet, consisting of diet PD (Jukes and Sanford, '39), 99.5 parts, and fish oil blend (3000-A, 400-D), 0.5 part. Birds which died during the first 9 days were omitted from the calculations since perosis seldom developed before the ninth day.

In the first series some natural foods were tested with the results indicated in table 1.

TABLE 1

CHANGES IN DIET 161	PER CENT OF BIRDS SHOWING PEROSIS AT:				
	12 days	14 days	19 days	29 days	33 days
None	36	64	82	100	100
Ground wheat replacing yellow cornmeal	40	70	80	80	80
15 parts alfalfa meal replacing 15 parts yellow cornmeal	36	64	64	73	73
5 parts dried pork liver replacing 5 parts casein	36	45	45	36	27
Normal diet replacing diet 161	0	0	0	0	0

Wheat and alfalfa possibly exerted a slight anti-perotic effect. The effect of alfalfa was so low that vitamin K was excluded as an anti-perotic substance. In the case of liver, there was a tendency for some of the milder cases to retrogress after the third week.

Recently Hogan, Richardson and Patrick ('40) announced that liver contained a water-soluble organic factor which prevented perosis in chicks. This prompted an examination of the known B-vitamins. A mixture ("synthetic vitamin mixture") was made which included thiamin, 1.2 mg.; riboflavin, 1.8 mg.; nicotinic acid, 3 mg.; pyridoxine (vitamin B_6), 0.9 mg.; and pantothenic acid, 10 mg. To this mixture was added choline chloride, 0.15 gm., since this substance had been observed to stimulate growth slightly in chicks (Jukes, '40 b). The results are summarized in table 2.

The synthetic mixture completely protected against perosis. Sardine meal also appeared to supply a small amount of a protective factor which caused retrogression of mild cases after the third week, as was observed in the case of liver in the first series.

In the next experiment, thiamin, nicotinic acid, pantothenic acid and choline were tested separately. Only choline was effective. This experiment was previously reported (Jukes, '40 a). Some other supplements were tested in the same series with the results indicated in table 3.

TABLE 2

CHANGES IN DIET 161	PER CENT OF BIRDS SHOWING PEROSIS AT:				
	12 days	14 days	19 days	24 days	28 days
None	33	44	67	78	89
Synthetic vitamin mixture plus choline, as above, added to 100 gm. diet	0	0	0	0	0
17 parts sardine meal replacing casein and $CaCO_3$	20	60	70	30	10
Normal diet replacing diet 161	0	0	0	0	0

TABLE 3

SUPPLEMENT TO 100 GM. OF DIET 161	PER CENT OF BIRDS SHOWING PEROSIS AT:			
	12 days	14 days	19 days	28 days
None	71	71	85	85
Barley replacing cornmeal	12	12	12	38
2 gm. fish oil blend (3000-A, 400-D)	40	50	60	60
10 gm. gum arabic + 5 gm. gelatin	43	57	71	86
10 gm. dehydrated cane molasses	25	25	25	50
Normal diet replacing diet 161	0	0	0	0

At this time it was noted that there was no mortality during the first week in the case of a group of birds receiving supplementary thiamin. In succeeding experiments, diet 161 was modified by the addition of 50 μg. of thiamin to 100 gm. of diet.

Table 3 shows that barley supplied a small amount of the protective factor, but that additional vitamins A and D had no effect. A combination of gum arabic and gelatin, shown by Almquist, Mecchi, Stokstad and Manning ('40) to supply factors essential for the growth of chicks, did not prevent

perosis. Cane molasses was fed as a source of biotin. A sample of the molasses was kindly assayed by Dr. E. E. Snell and found to contain 2.6 µg. of biotin per gram. The preventive action of the molasses was very low.

In the next experiment a number of synthetic substances were tested, including betaine, which has been shown to have certain biological activities similar to those of choline (Best and Huntsman, '32; du Vigneaud et al., '39). Arseno-choline[1] was tested for the same reason (Welch, '36). The results are in table 4.

TABLE 4

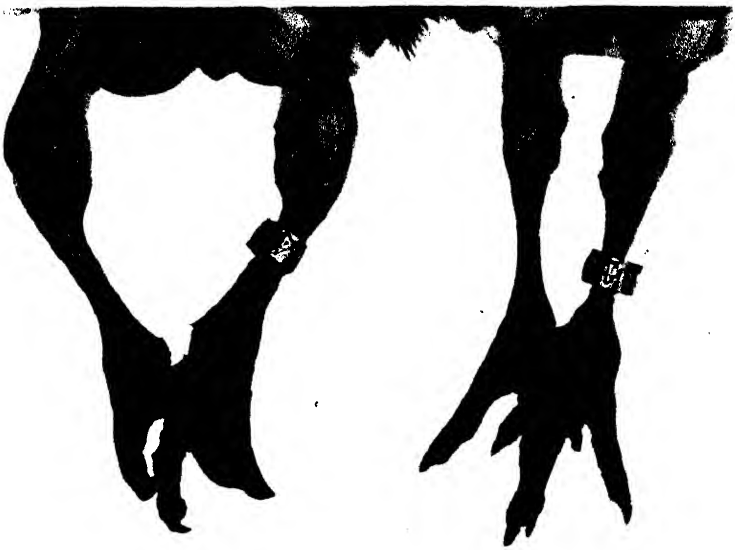
SUPPLEMENT TO 100 GM. OF DIET 161	PER CENT OF BIRDS SHOWING PEROSIS AT:				
	11 days	15 days	21 days	23 days	28 days
None	20	20	50	50	50
Synthetic vitamin mixture as above, without choline	25	42	58	50	50
0.2 gm. choline	0	0	0	0	8
Synthetic vitamin mixture with 0.2 gm. choline	0	0	0	0	0
0.2 gm. choline + 24 mg. pantothenic acid.	0	0	9	9	0
24 mg. pantothenic acid	50	40	60	60	60
0.9 mg. pyridoxine	18	55	64	64	82
0.2 gm. arsenocholine	0	9	9	9	9
0.5 gm. betaine	64	82	82	82	82
25 gm. rice bran replacing 25 gm. corn	11	11	22	22	11
25 gm. wheat bran replacing 25 gm. corn	25	33	58	50	50
25 gm. soybean meal replacing 12 gm. corn and 13 gm. casein	9	0	0	0	0
Normal diet replacing diet 161	0	0	0	0	0

The weights refer to choline and arseno-choline as the chlorides, and to betaine as the hydrochloride in all experiments in which these compounds were used. The results indicated that choline alone of the components of the synthetic mixture was able to prevent perosis. Arseno-choline was definitely effective, although one mild case of perosis appeared. Betaine was completely ineffective at the level fed. Of the natural foods, soybean meal was a relatively good source of the

[1] Kindly supplied by Dr. A. D. Welch.

preventive factor, rice bran was a poor source, and wheat bran was ineffective. Figure 2 illustrates a case of perosis from this series.

In the preceding experiments it was observed that choline as a sole supplement was effective in preventing the gross symptoms of perosis. However, when choline was fed in combination with the synthetic vitamin mixture, the leg bones



a b

Fig. 2 Perotic hock joints (a) of a turkey receiving betaine (see table 4) compared with normal hocks (b) of a turkey receiving choline as a supplement to diet 161. The birds were 21 days old when photographed, and weighed 107 and 147 gm. respectively.

were more normal in appearance than when choline was fed alone. This led to the adoption of a more "simplified" basal diet, containing yeast as a source of the vitamin B complex. It was found that choline prevented perosis and also promoted growth when fed as a supplement to this diet (Jukes, '40 a) which consisted of glucose,[2], 50 parts; washed casein, 30; dried

[2] "Cerelose."

yeast,[3] 10; salt mixture (Jukes, '39 b), 5; soybean oil, 4; and fish oil blend, 1 (diet 162). The diet contained approximately 166 parts of manganese per million parts of diet. The results shown in table 5 were obtained.

The results in table 5 indicate that 0.1% of choline was a sufficient supplement for growth but was not enough to prevent perosis completely. There was a tendency for some of the milder cases to retrogress in this group during the latter half of the experimental period. This recalls the preceding results with partially protective diets. The addition of 0.2% of

TABLE 5

Incidence of perosis and growth on a simplified diet

SUPPLEMENT TO 100 GM. OF DIET 162	PER CENT OF BIRDS SHOWING PEROSIS AT:					WEIGHT IN GRAMS AT:			
	10 days	12 days	13 days	15 days	28 days	7 days	15 days	19 days	28 days
None	27	36	55	73	82	62	90	110	155
0.1 gm. choli	8	8	33	33	17	62	120	158	211
0.2 gm. choli	0	0	0	0	0	62	110	137	209
0.1 gm. choline + 5 gm. gum arabic + 5 gm. gel.	0	11	22	33	33	62	114	154	211
0.3 gm. betaine	25	62	75	88	100	61	84	106	139
20 gm. rice bran	50	70	80	70	80	68	109	143	189
Normal diet replacing diet 162	0	0	0	0	0	65	119	159	208

choline completely protected against perosis and produced bones of normal appearance. Betaine was ineffective and the symptoms in the birds receiving betaine were more acute than in the birds on the basal diet. Gum arabic and gelatin did not produce additional growth, thus suggesting a species difference between chicks (Almquist et al., '40) and turkeys. Twenty per cent of rice bran improved growth but was ineffective in preventing perosis. This is in disagreement with the result obtained with rice bran on diet 161. Perhaps this was due to diet 162 being more deficient than diet 161, and

[3] Anheuser-Busch, strain G.

hence the slight anti-perotic activity of rice bran was too weak to become apparent in the case of the more deficient diet.

In another experiment a group of nine turkeys were injected weekly with 0.1 gm. inositol, which was the level used by Wiese et al. ('38) in experiments with chicks, and reported by them to prevent perosis. However, perosis developed in the injected turkeys at approximately the same rate as in the case of birds on the basal diet.

AN EXPERIMENT WITH CHICKS

The preceding observations naturally raised the question of the possibility of an effect of choline on perosis in chicks. A somewhat different diet was devised to test this point.

TABLE 6

Effect of choline on growth and perosis in chicks

SUPPLEMENT TO 100 GM. OF BASAL DIET 109	PER CENT OF BIRDS SHOWING PEROSIS AT:				WEIGHT IN GRAMS AT:			
	12 days	17 days	25 days	32 days	8 days	17 days	25 days	32 days
None	33	33	50	75	55	78	116	145
0.1 gm. choline	0	0	0	9	54	95	141	175

Sodium alginate [4] and gelatin were added to supply the "rice factor" (Almquist et al., '40). Synthetic vitamins were used to replace part of the yeast. The diet had the following percentage composition (diet 109): glucose,[5] 53 gm.; washed casein, 25; gelatin, 5; sodium alginate,[4] 5; soybean oil, 5; dried yeast,[6] 5; salt mixture (Jukes, '39 b), 6; fish oil blend, 0.3; thiamin, 0.6 mg.; riboflavin, 0.8; nicotinic acid, 1.5; pyridoxine, 0.6; pantothenic acid, 3.0. Twelve Single Comb White Leghorn chicks were used in each group. The results are summarized in table 6, and indicate that choline is essential for the prevention of perosis in chicks.

[4] "Kelgin."

[5] See footnote 2.

[6] See footnote 3.

Growth was promoted by choline, but the stimulatory effect was less in the case of the chicks than was observed with turkeys (table 5).

DISCUSSION

Dietary choline has been demonstrated to have the following actions: lipotropic (Best et al., '32); protective against hemorrhagic degeneration of the kidney (Griffith and Wade, '39); and functioning in the conversion of homocystine to methionine (du Vigneaud et al., '39). In all three cases, betaine has an action similar to that of choline (Best and Huntsman, '32; Welch, '40; du Vigneaud et al., '39). This might indicate that choline is the precursor of betaine and that betaine, formed from choline in vivo, is actually responsible for the physiological activity of dietary choline. However, it has been suggested by Welch and Welch ('38) that betaine functions in this regard as a precursor of choline rather than the reverse being true. The complete lack of growth-promoting and anti-perotic activity of betaine indicates that the turkey is unable to convert betaine to choline to an appreciable extent. This further suggests that betaine is not formed from choline in vivo as a necessary step for the physiological action of choline, and that in cases where dietary betaine is active, the activity is due to the ability of the experimental animals (rats) to convert betaine to choline. Evidently turkeys do not possess this ability to an effective degree.

No biochemical explanation of perosis has yet been advanced. Perosis in chicks has been found to be associated with a slight lowering of the ash content of the bones (Caskey et al., '39). It has also been stated (Wiese et al., '38) that the phosphatase content of the blood and bones of perotic chicks is lower than normal. The present investigation shows that choline is necessary for normal bone formation in chicks and turkeys. Choline is known to accelerate the formation of phospholipids in the liver, following which the phospholipids are rapidly removed from this organ (Perlman and Chaikoff, '39). This observation links choline with phosphorus metabolism. The formation of

phospholipids would presumably be accompanied by increased esterification of phosphoric acid. This might lead to a drain upon the supply of inorganic phosphate. It is interesting to note that Nielsen and Madsen ('40) reported that the serum inorganic phosphorus of perotic turkeys was higher than normal.

It is possible to speculate upon the question of the identity of choline with the anti-perotic factor in feeding stuffs. Choline is present in lecithin and other phospholipids, and possibly also may be present in other forms (Thannhauser et al., '39). Some data are available on the lecithin content of some natural materials. According to data cited by Horvath ('36), soybeans contain from 1.6 to 3.0% of phosphatides, and 38% of the phosphatide content is lecithin. A relatively small proportion of the phosphatides is removed by extraction of the oil by the usual methods (Horvath, '37). Soybean meal may hence be expected to contain about 0.6 to 1.1% of lecithin, which represents a content of about 0.08 to 0.15% of choline in soybean meal. The observed anti-perotic activity of soybean meal, noted also by Funk and Kempster ('40), may therefore be at least partially attributable to its choline content. Sardine meal, which had some preventive effect, would presumably furnish appreciable amounts of lecithin. Bähr and Wille ('31) reported that fish meal, prepared from whole fish, contained from 1.0 to 2.5% of "lecithin." Dried pork liver (table 1) was also observed to have some anti-perotic activity. Bloor ('28) reported the average lecithin content of fresh beef liver to be 1.6%. This would correspond to about 0.6% of choline combined as lecithin in dried beef liver.[7] Dried skim milk, used in basal diet 161, obviously had little or no anti-perotic activity. It is also low in lecithin. Heinemann ('39) reported that dried skim milk contains about 0.16% of lecithin.

Fresh egg yolk contains about 10% of phospholipids, and a sample of phospholipids prepared from egg yolk was found to consist of about 80% of lecithin (Jukes, '34). A consider-

[7] Fletcher et al. ('35) reported 0.27% of choline in beef liver, and 0.09% in dried skim milk.

able portion of the yolk is retained in the yolk sac within the body of the newly-hatched bird. The results in table 5 show that the birds on the basal diet grew at the same rate as the birds on the diets supplemented with choline in the first week, after which a difference appeared in the rate of growth. This would indicate that the turkeys were utilizing a reserve supply of choline during the first week. In this connection it may be noted that the yolk sac of the newly hatched chick contains 1.6% of phospholipid, nearly all of which is absorbed by the chick during the first week (Entenman et al., '40).

SUMMARY

1. The addition of choline to a deficient basal diet, containing added manganese, prevented perosis in turkeys. Arsenocholine was also anti-perotic, but betaine was completely ineffective.

2. A number of vitamins and minerals were tested and all were found ineffective. However, an adequate supply of the vitamin B complex in the diet was found to be necessary for the full anti-perotic effect of choline to be exerted.

3. The anti-perotic effect of various feeding stuffs was studied. Soybean meal was fully effective at a level of 25%. Partial protection was obtained with either 17% of sardine meal, 5% of dried pork liver, or with 65% of barley. Some other feeds had a slight potency. The relation of the lecithin content to the anti-perotic activity of certain feeds is discussed.

4. Experiments with a simplified diet indicated that choline is essential for growth as well as for the prevention of perosis. An addition of 0.1% of choline was sufficient for growth, but insufficient for the prevention of perosis. An addition of 0.2% of choline protected against perosis. Betaine was neither growth-promoting nor anti-perotic. Inositol was ineffective.

5. The addition of a supplement to supply the "rice factor" did not promote growth in turkeys, which may indicate a difference between this species and the chick.

6. An experiment with chicks indicated that choline is essential for the prevention of perosis in this species also.

7. Biochemical possibilities arising from these observations are discussed briefly.

The assistance of Robert E. Ranney and Phillip Levy is gratefully acknowledged. Thiamin, riboflavin, nicotinic acid and pyridoxine were kindly supplied by Merck and Company. Dried yeast was generously furnished by Anheuser-Busch, Inc., and washed casein by the Golden State Company.

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THE DISTRIBUTION OF THE "GRASS JUICE FACTOR" IN PLANT AND ANIMAL MATERIALS [1]

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ONE FIGURE

Previous publications from this laboratory have shown that young grass contains a water-soluble growth-promoting substance (or substances) for the rat (Kohler et al., '36, '37) and the guinea pig (Kohler et al., '38). It has also been shown that this factor is distinct from all other known nutritional essentials (Kohler et al., '37, '38). Cows grazing on good pastures or fed good quality roughage can transmit this factor to their milk (Johnson et al., '39). Johnson et al. have also shown that the grass juice factor can be preserved through ensiling processes. The guinea pig has proved to be a more sensitive test animal than the rat in assaying for this factor (Kohler et al., '38). It was found that guinea pigs fed mineralized winter milk alone lost weight and died within 3 to 10 weeks, whereas those animals receiving the same milk supplemented with small amounts of grass or grass juice grew at a normal rate.

Kohler, Randle and Wagner ('39 a) showed that the "grass juice factor" was precipitated from grass juice by the addition of acetone and could be removed from this precipitate by wash-

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the American Butter Company, Abbott Laboratories, and the Works Progress Administration.

[2] Now with Cerophyl Laboratories, Kansas City, Missouri.

ing with acid acetone. A chloroform-amyl alcohol mixture removed the proteins from the grass juice and left the factor in the aqueous layer. When this deproteinated juice was treated with norite, the filtrate retained practically all of the original activity. The best concentrates were active when fed at a level of 40 mg. of solids per day. This represented a fiftyfold concentration.

Cannon and Emerson ('39) have reported on the dietary requirements of guinea pigs on purified diets. These diets, although supplemented with the other known vitamins, failed to support growth. They reported that supplements of lettuce or grass to these diets would give normal growth for considerable periods and believed their factor to be identical with the "grass juice factor." However, the growth they obtained was markedly inferior to that obtained in this laboratory when the winter milk diet or purified diet (Kohler et al., '39 b) was supplemented with grass.

This paper presents a study of the distribution of the "grass juice factor" in various plant and animal tissues.

EXPERIMENTAL

The feeding technique described by Kohler, Elvehjem and Hart ('38) was used. Fresh unpasteurized winter milk, obtained from cows maintained throughout the year on a ration of grain, corn silage and dry hay, was supplemented with a mineral mixture supplying 1 mg. of iron as ferric pyrophosphate, 0.1 mg. of copper as copper sulfate and 0.1 mg. of manganese as manganese sulfate per day. Unless otherwise stated, all the food materials used in these assays were obtained fresh, chopped in a food chopper, dried in a drying room (35 to 40°C.), ground, and stored in a refrigerator (5 to 10°C.) until fed. The food materials were fed to guinea pigs (275 to 325 gm.) as supplements to a small amount of milk in the morning. Later in the day, after the supplement was consumed, a larger dish of milk was placed in the cage to insure ad libitum feeding. The animals were kept in individual wire cages with raised screen floors.

In the earlier studies on the "grass juice factor" in this laboratory, orange juice was fed as a source of extra ascorbic acid. Later experiments (Kohler et al., '38) showed that guinea pigs consuming from 80 to 200 cc. of milk showed no improvement with the addition of orange juice. This is consistent with the finding that milk contains 2 to 2.5 mg. of vitamin C per 100 cc. and that 0.3 mg. of pure ascorbic acid prevents scurvy and supports good growth in the guinea pig. Animals live from 3 to 10 weeks on the basal milk diet, and have never shown any signs of scurvy upon autopsy.

DISCUSSION

Typical growth curves for animals receiving mineralized winter milk alone and the basal diet plus certain supplements are shown in figure 1. Curve 629 is the usual type for animals

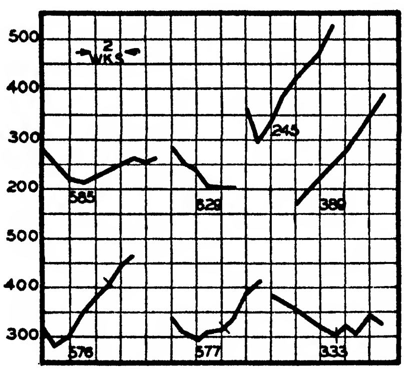

Fig. 1 Growth curves of guinea pigs fed mineralized winter milk plus 2 gm. of the supplement as follows:

585♂—Control
629♂—Control
245♀—Rye grass
389♀—Cerophyl no. 71921
576♂—Peas
577♂—Canned peas
333♂—Carrots
\ = supplement raised to 3 gm. per day.

receiving the basal ration; however, occasionally an animal is able to maintain itself for several weeks (curve 585). Under these experimental conditions, 2 gm. per day of a supplement rich in the grass juice factor promotes excellent growth in

guinea pigs (curves 245 and 389). Three grams per day does not increase the growth rate significantly over that of the 2 gm. level (curve 576). An increase in the amount fed per day may stimulate additional growth if the supplement is only a fair source of this factor (curve 577). Higher levels of a supplement that is poor in the "grass juice factor" do not increase the daily growth rate significantly (curve 333). It should be pointed out that there is usually a temporary loss in weight when the animals are shifted from the stock ration to the supplemented winter milk diet (fig. 1). Therefore, the average daily gain for the animals has been calculated on a 6 weeks' basis beginning with the second week.

A considerable number of plant tissues have been assayed for the "grass juice factor." The following have been found to be the best sources: dehydrated cereal grass,[3] rye grass, young white clover, peas, pea shells, cabbage, turnip tops, spinach, etc. The materials poorest in this factor were apples, celery, molasses, peanuts, turnips, lettuce, oats, etc. Such tissues as young berries, cauliflower, canned peas, beans, etc., were found to be intermediate sources of the "grass juice factor."

The data of this paper and other papers from this laboratory indicate that cereal grasses are excellent sources of the "grass juice factor." The results reported here support the observation made by Kohler et al. ('38) that the potency of the grasses as a source of this factor varies with the age of the plant, i.e., the small rapidly metabolizing plant is much richer than the older and more mature plant. The "grass juice factor" activity varies considerably from one plant to another (table 1). In addition, the amount of this factor in any one given plant material may also show a great variation.

Some of the crystalline vitamins and highly purified vitamin concentrates have been tested to determine whether they show a supplementary growth effect above that of the "grass juice factor." The following crystalline vitamins did not further stimulate growth in guinea pigs on a milk diet: ascorbic acid

[3] Cerophyl, supplied by the Cerophyl Laboratories, Kansas City, Missouri.

TABLE 1

Distribution of the grass juice factor in plant and animal tissues [1]

NO.	ANIMAL NO.	FOODSTUFF	LEVEL FED	AVERAGE WEIGHT GAIN	REMARKS
			gm./day	*gm./day*	
1	629	Control [2]	0	—1.3	
2	585	Control [2]	0	0.2	
3	245	Rye grass [2]	2	5.3	
4	389	Cerophyl [2]	2	4.9	
5	285	Clover, white	2	4.0	Young, 6–8 inches tall
6	286	Clover, white	2	2.0	Older, 8–10 inches tall
7	576	Peas	2–3 [3]	3.9	
8	601	Peas, frozen	2	4.0	Commercial
9	577	Peas, canned [2]	2–3 [3]	1.8	
10	575	Peas, pods	2–3 [3]	3.0	
11	605	Peas, juice	20 cc.	1.9	
12	604	Peas, shells	3	4.0	
13	607	Peas, pulp	2	1.1	Residue after pressing juice from peas
14	570	Bean, Ky. Wonder	2–3 [3]	3.0	
15	572	Beans, soy	2–3 [3]	3.0	Whole green beans
16	236	Carrots	2	3.0	
17	333	Carrots [2]	2	—0.8	
18	455	Young berries	2	1.3	
19	456	Young berries	13.3	1.4	Fed in fresh state
20	600	Lettuce	2	1.5	
21	599	Cabbage	2–3	4.0	
22	490	Turnips	2	0.8	
23	438	Turnip tops (greens)	2–3	3.9	
24	582	Spinach	2–3	3.6	
25	583	Cauliflower	2–3	3.3	Heart
26	584	Cauliflower	2–3	1.6	Outer leaves
27	400	Peanuts	2–5	1.1	
28	493	Apples	2	1.0	
29	478	Molasses (blackstrap)	4 cc.	0.6	
30	581	Celery	2–3	0.8	
31	621	Brewers' yeast	4	2.0	
32	409	Hulled oats	4	2.1	
33	552	Rolled oats	4	—2.0	
34	215	Sprouted oats	3	3.7	
35	263	Rice bran	2	2.3	
36	367	Wheat germ	2–4	1.4	
37	218	Brain	1	1.3	Dried
38	182	Egg white	2–5	0	
39	217	Egg yolk	2	—0.7	
40	535	Beef heart	2	—0.5	
41	620	Liver extract powder	2	0	Wilson's 1–20 powder [4]
42	617	Whole milk powder	2	—0.8	Klim
43	611	Skim milk powder	2.5	—8	
44	616	Whey powder	2	1.2	
45	207	Cerophyl no. 825—3 + 0.001 M KCN	2	4.9	
46	204	Cerophyl no. 825—3 + 1% H_2O_2	2	1.0	
47	208	Cerophyl no. 825—3 + NaOH	2	2.9	
48	206	Cerophyl no. 825—3	2	3.3	
49	205	Cerophyl no. 825—3 + H_2O	2	4.8	
50	209	Cerophyl no. 825—3 (ashed)	≎ 2	—1.6	

[1] The data given in this table represent the results of a typical response of one animal on each level of sample assayed. The actual number of animals used in each assay varied from one to six.

[2] Growth curves for animals receiving these supplements are included in figure 1.

[3] This indicates that the level of the supplement was raised during the course of the experiment.

[4] From the Wilson Laboratories, Chicago.

10 mg. per day, riboflavin 50 and 100 γ per day, vitamin B_6 50 γ per day, vitamin E 1 mg. per week, thiamin 500 γ per day and nicotinic acid 12.5 mg. per day. Highly purified concentrates of pantothenic acid (150 γ per day) and vitamin P preparations (citrin, hesperidin, and calcium eriodictate)[4] 1 mg. per day similar to those of Rusznyak and Szent-Gyorgyi ('36) failed to promote growth in the guinea pig on the basal ration.

Euler and Malmbery ('34) has reported the presence of an anti-pneumonia factor for guinea pigs in berries such as black currants, mountain ash berries, etc. Fresh frozen young berries [5] (both fresh and dry) were therefore investigated as a source of the "grass juice factor." They produced some growth (table 1).

Rygh and associates ('32) believed that in addition to ascorbic acid, narcotine or nor-narcotine was essential for the prevention of scurvy in the guinea pig. Since these alkaloids had been isolated from scorbutic rations, fruits and plant materials, narcotine hydrochloride [6] was fed to several animals at 30, 60 and 120 μg. per day without stimulation of growth. In one case, however, the animal on being raised to the 60 μg. level grew 3 gm. per day for 6 weeks, then exhibited a decline and died 5 weeks later. The growth in this case may have been due to a temporary stimulating effect of the alkaloid. Later ('38–'39) Rygh reported that glucuronic acid and narcotine prevented scurvy in guinea pigs. Ten milligrams of glucuronic acid [7] per day had no beneficial effects on guinea pigs deficient in the "grass juice factor"; a combination of glucuronic acid and narcotine was also ineffective.

Kohler, Elvehjem and Hart ('38) reported that the "grass juice factor" was labile to autoclaving or storage at room

[4] The citrin, hesperidin and calcium eriodictate were kindly supplied by Dr. C. Nielsen, Abbott Laboratories, North Chicago, Illinois.

[5] Birdseye brand.

[6] The narcotine hydrochloride was kindly supplied by Dr. M. H. Seevers, Department of Pharmacology, University of Wisconsin.

[7] The glucuronic acid was kindly supplied by Dr. K. P. Link, Department of Biochemistry, University of Wisconsin.

temperature. Further studies were made on the stability of this factor in grass. Three 120-gm. samples of dried grass (cerophyl no. 825-3) were weighed into beakers. One sample was treated with 300 cc. of 0.001 M potassium cyanide, another with 300 cc. of 1% hydrogen peroxide, and the third with cold sodium hydroxide solution, pH 9. The samples were allowed to stand at room temperature for 30 minutes. At this time the sodium hydroxide treated sample was neutralized and all three samples were dried at 35 to 40°C. for 24 hours. An untreated sample and a water-treated sample were fed at the same time as positive controls. Each sample was fed at a 2-gm. level. Still another portion of the grass was ashed, the ash suspended in water, and fed at the equivalent of the 2-gm. level. The water, potassium cyanide, and the sodium hydroxide treated samples were as effective in promoting growth as the untreated sample of grass. Hydrogen peroxide treatment destroyed practically all of the activity and the ashed sample retained none of the original activity. The destruction of the "grass juice factor" appears to be an oxidative process.

SUMMARY

The relative distribution of the "grass juice factor" in plant and animal tissues is reported. Animal tissues appear to be a poor source of the factor. The amount present in plant materials varies considerably. The young rapidly metabolizing plant tissues are much richer in this growth promoting substance than the older and more mature plant material. Much of the original activity can be retained by careful drying and storage at low temperatures. The factor is labile to oxidation. Glucuronic acid, narcotine, citrin and specific fractions of citrin, i.e., hesperidin, and calcium eriodictate, do not have the activity of the "grass juice factor."

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THE INFLUENCE OF LACTOSE ON CALCIUM RETENTION IN CHILDREN [1]

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The ability of lactose, the sugar of milk, to influence calcium metabolism or the ash content of body tissue has been demonstrated in several species—the dog (Dragstedt and Peacock, '23; Inouye, '24; French and Cowgill, '37), the rat (Bergeim, '26; Mitchell, Hamilton and Beadles, '37; Outhouse, Smith, Merritt and White, '37; Outhouse, Smith and Twomey, '38), the calf (Robinson, Huffman and Masor, '29), the chick (Kline, Keenan, Elvehjem and Hart, '32) and the pig (Whittier, Cary and Ellis, '35). In this laboratory, the increase observed in the total ash content of bones and in the calcium and phosphorus storage as a result of lactose-feeding was not as great as that obtained when vitamin D was administered in the form of cod liver oil. Kline, Keenan, Elvehjem and Hart ('32), who added lactose to the diet of irradiated chicks, suggested that a combination of this nutrient and vitamin D was more efficacious than either one administered alone; they believed that lactose might act as a supplement to vitamin D in promoting calcium assimilation. Such findings carry interesting implications as to the possibility of increasing calcium utilization in man—particularly since it has been shown that man is not economical in his use of dietary calcium (Steggerda and Mitchell, '39; Kinsman, Sheldon, Jensen, Bernds, Outhouse and Mitchell, '39; Kempster, Breiter, Mills, McKey, Bernds

[1] Aided by a grant from the American Dry Milk Institute, Inc., Chicago, Illinois.

and Outhouse, '40). To test this possibility, an experiment was devised which would show the effect, if any, of lactose on calcium metabolism in children; this paper is a report of that investigation.

EXPERIMENTAL

The subjects of this experiment were five little boys, four of whom had participated in the previously reported work on the utilization of the calcium of milk (Kinsman, Sheldon, Jensen, Bernds, Outhouse and Mitchell, '39) and of $CaHPO_4$ (Kempster, Breiter, Mills, McKey, Bernds and Outhouse, '40). Their ages, heights and weights are given in table 1. The basal dietary was the same as that of the previous studies; although it provided generous quantities of fruits, meat and eggs, it contained milk solids equivalent to only 200 cc. of fluid milk and, hence, was poor in calcium. Approximately 1000 I.U. of vitamin D in the form of cod liver oil were fed daily—and had been fed daily for many weeks prior to the beginning of the experiment—in order to provide optimal conditions for the utilization of calcium.

The source and quantity of calcium received by the five subjects during this experiment were essentially the same as those of period VI in the study on the utilization of the calcium of $CaHPO_4$. In fact, period VI, in which $CaHPO_4$ was given as a supplement to the basal dietary, constitutes the control period of the present study. (Inasmuch as subject D stored such small amounts of calcium during this period, a second control period, period IX, had to be run on this child.) During a subsequent period (period VIII) in which lactose was fed, this phosphate was administered but in a greater quantity; the increase was necessary in order to equalize the total intake of calcium for the two periods since the children had shown a tendency, during period VII,[2] toward a decrease in the consumption of ad libitum foods. The $CaHPO_4$ contributed 126 mg. of calcium during the 7 weeks of period VI

[2] Period VII was the basal period for the previously reported study on the utilization of diphosphate calcium and is not essential to the present report.

TABLE 1

Calcium metabolism data (ave. daily) during lactose and non-lactose feeding

Subject W — Age in yrs., 4.8 — Weight in kg., 20.3 — Height in cm., 108.6

PERIOD AND WEEK		INTAKE	OUTPUT Urinary	OUTPUT Fecal	RETENTION
		mg.	mg.	mg.	mg.
VI	2[1]	479	37	338	104
	3	483	46	324	113
	4	509	58	323	128
	5	501	56	280	165
	6	497	60	296	142
	7	488	64	292	131
	Ave.	493	54	309	131
VIII	2	520	46	335	139
	3	511	58	280	173
	4	515	50	312	154
	5	514	55	317	142
	6	516	58	309	149
	Ave.	515	53	311	151

% retention: period VI, 26.6; period VIII, 29.3. Increase in % retention, 10.2%.

Subject C — Age in yrs., 5.9 — Weight in kg., 22.6 — Height in cm., 117.3

PERIOD AND WEEK		INTAKE	OUTPUT Urinary	OUTPUT Fecal	RETENTION
		mg.	mg.	mg.	mg.
VI	2[1]	495	52	337	106
	3	504	57	356	90
	4	543	66	340	137
	5	523	75	344	104
	6	508	64	355	90
	7	496	72	334	91
	Ave.	512	64	344	103
VIII	2	522	60	351	112
	3	515	61	314	140
	4	516	57	411	48
	5	517	46	347	123
	6	517	35	351	131
	Ave.	517	52	355	111

% retention: period VI, 20.1; period VIII, 21.5. Increase in % retention, 7.0%.

Subject Je — Age in yrs., 7.0 — Weight in kg., 20.7 — Height in cm., 124.1

PERIOD AND WEEK		INTAKE	OUTPUT Urinary	OUTPUT Fecal	RETENTION
VI	2	483	94	313	76
	3	489	123	303	63
	4	513	167	269	77
	5	493	139	308	47
	6	490	126	294	70
	7	484	151	265	79
	Ave.	492	133	292	69
VIII	2	518	110	271	137
	3	512	96	273	143
	4[2]	482	138	268	76
	5	520	95	320	106
	6	520	125	344	51
	7	514	108	286	120
	8	515	105	321	89
	9	518	85	314	119
	Ave.	517	103	304	109

% retention: period VI, 14.0; period VIII, 21.1. Increase in % retention, 50.7%.

Subject Br — Age in yrs., 6.8 — Weight in kg., 26.4 — Height in cm., 121.5

PERIOD AND WEEK		INTAKE	OUTPUT Urinary	OUTPUT Fecal	RETENTION
VI	2	496	83	332	81
	3	511	118	297	97
	4	544	140	339	64
	5	523	109	320	95
	6	507	103	314	89
	7	488	101	319	69
	Ave.	512	109	320	83
VIII	2	520	90	293	136
	3	515	67	300	148
	4[2]	520	64	303	153
	5	533	66	283	185
	6	526	42	305	179
	Ave.	523	66	297	160

% retention: period VI, 16.2; period VIII, 30.6. Increase in % retention, 88.9%.

Subject W

PERIOD AND WEEK		INTAKE	OUTPUT Urinary	OUTPUT Fecal	RETENTION
VII	2	346	65	171	110
	3	347	46	168	133
	4	357	81	216	60
	5	364	68	200	106
	6	369	79	167	123
	7	377	68	198	111
	8	355	50	195	109
	Ave.	359	65	188	107
X	2	341	50	191	100
	3	356	41	217	99
	4	349	34	235	80
	5	345	48	194	102
	Ave.	348	43	209	95

% retention: period VII, 29.8; period X, 27.3. Decrease in % retention, 8.4%.

Subject C

PERIOD AND WEEK		INTAKE	OUTPUT Urinary	OUTPUT Fecal	RETENTION
VII	2	356	70	249	37
	3	364	77	225	62
	4	362	71	229	62
	5	371	63	256	52
	6	366	62	216	88
	7	376	69	218	89
	8	351	52	204	96
	Ave.	364	66	228	69
X	2	360	34	219	107
	3	375	29	199	147
	4	354	37	239	78
	5				
	Ave.	363	33	219	111

% retention: period VII, 19.0; period X, 30.6. Increase in % retention, 61.0%.

Subject D. Age in yrs., 6.1. Weight in kg., 19.4. Height in cm., 111.2.

Week	Control period IX Intake	Urinary	Fecal	Retention	Lactose period VIII Intake	Urinary	Fecal	Retention
2[1]	517	56	312	149	484	55	311	117
3	519	53	338	128	500	41	281	179
4	515	59	299	156	505	46	305	154
5	514	63	332	119	492	48	307	138
6	513	54	292	167	500	47	312	141
7	529	41	370	118				
8[2]	487	47	376	63				
9	511	53	337	121				
Ave.	517	54	326	137	496	47	303	146

% retention: period IX, 26.5; period VIII, 29.4. Increase in % retention, 10.9%.

[1] Data omitted for the first week following a change in the level of calcium intake.
[2] Subject ill; data not included in average.

and 176 mg. during the 6 to 9 weeks of period VIII. The total daily calcium intake for both periods approximated 500 mg.; this level was selected because, in previous studies, it had induced, in these same children, less than maximal retention of calcium. In studies on calcium utilization, the intake level is important inasmuch as the full effect of any substance could not be discerned if the subjects were already storing maximal quantities of calcium. As a matter of fact, as the experiment progressed it was suspected that subjects W and C were receiving too much calcium; therefore, during a subsequent short period (period X) they were given lactose in conjunction with the basal dietary. The retentions observed during this period were compared with those of period VII during which the basal dietary alone was fed.

Thirty-six grams of lactose supplemented the dietary during period VIII. This amount of the carbohydrate plus that of the milk solids of the basal dietary approximated the quantity of lactose contained in 1 quart of milk. The milk sugar was obtained from the same source as was that used in an earlier study of the influence of lactose on bone calcification in the rat (Outhouse, Smith, Merritt and White, '37); it was ash-free and contained no sterols. During this period, the quantity of sucrose which the children had been allowed in their basal diet was decreased by an amount equivalent to the added lactose so that the intake of di-saccharides might remain constant.

The procedures used in the collection of the metabolic materials and in the analysis for calcium corresponded exactly with those of the earlier experiments and need not be discussed here.

RESULTS

The children remained healthy, active and cooperative throughout the experiment and made satisfactory gains in weight. Their appetites were good, and ennui was never noted. During the period in which lactose was fed, no effect—adverse or otherwise—on intestinal motility was discerned.

The metabolic data are given in table 1. During the period in which $CaHPO_4$ was the only supplement to the basal dietary

(i.e., period VI for four subjects and period IX for subject D), the daily calcium intakes for subjects W, D, C, Br and Jw were 493, 517, 512, 512 and 492 mg., respectively; these values averaged 505 mg. At these levels of intake, the average quantity of calcium stored each day by the individual children, in the order listed above, was 131, 137, 103, 83 and 69 mg., respectively. These retentions amount to 26.6, 26.5, 20.1, 16.2 and 14.0% of the intake; the average for the group is 20.7%.

During period VIII when lactose was fed, the average daily intakes of calcium were 515, 496, 517, 523 and 517 mg., and the corresponding retentions were 151, 146, 111, 160 and 109 mg. for subjects W, D, C, Br and Jw. Expressed in terms of the per cent of the intake retained, these values become 29.3, 29.4, 21.5, 30.6 and 21.1 and average 26.4.

While subjects W and C were on the basal dietary (i.e., during period VII), they consumed, respectively, 359 and 364 mg. calcium daily and stored 107 and 69 mg. or 29.8 and 19.0% of the intake. During period X when 36 gm. of lactose supplemented the basal dietary, the children ingested 348 and 363 mg. of calcium and showed corresponding retentions of 95 and 111 mg. which represented 27.3 and 30.6%, respectively, of the intakes.

In this particular experiment, the criterion used for determining the effect of lactose on calcium metabolism involved a comparison of percentage retentions of calcium during the lactose period and the non-lactose period. A more desirable criterion would have been a comparison of percentage utilization values for these two periods, but the formula used in previous studies (Kinsman, Sheldon, Jensen, Bernds, Outhouse and Mitchell, '39; Kempster, Breiter, Mills, McKey, Bernds and Outhouse, '40) could not be applied since the lactose may have affected not only the di-phosphate calcium but that of the basal ration also. Inasmuch as each child did not consume exactly the same amount of calcium during both periods, the data had to be expressed in terms of the percentage increase in the percentage of calcium retained. On such a basis, the lactose fed during period VIII is shown to have

induced better calcium storage in all of these children; the retention percentages for the non-lactose and lactose periods, respectively, were 26.6 and 29.3 for W, 26.5 and 29.4 for D, 20.1 and 21.5 for C, 16.2 and 30.6 for Br and 14.0 and 21.1 for Jw. Though some of these differences may seem small, they constitute increases over the lower values of 10.2, 10.9, 7.0, 88.9 and 50.7% for the subjects in the same order as listed above. This would mean that there was an average increase in the amount of calcium retained during the lactose period of 33.5%. During period X when lactose was fed in conjunction with the basal dietary, subject C retained 61.0% more calcium than he did previously on the same low calcium intake when lactose was not fed whereas subject W responded conversely —retaining slightly less calcium.

In table 1 the data on pathways of excretion of calcium reveal a decrease in urinary values for all children during the time of lactose ingestion. The average losses in urinary calcium amounted to 16.9 and 12.0% of the intake during the control and lactose periods, respectively—a decrease of 29% during the latter period. (Statistical treatment of these data give high enough odds—i.e., 143 to 1—to enable one to conclude that the difference between the values was due to the ingestion of lactose and not to chance.)

DISCUSSION

The response of the subjects to the supplement of a moderate quantity of lactose during period VIII was unanimous; greater retentions of calcium were obtained in all cases. The mean increment in percentage retention for the group was approximately one-third of the mean percentage retention for period VI. Other evidence, which is fully as convincing of the stimulating effect of lactose on calcium utilization, can be secured for four of the subjects (subject D joined the group later in the experiment), by comparing data of periods II, III and IV during which the sources or levels of intake of calcium differed from those of periods VI and VIII. In every one of these periods, all subjects but one (Jw retained a higher percentage of calcium during one of these non-lactose periods)

retained an appreciably smaller percentage of calcium than they did later when they received lactose. If all the data for the three periods are averaged for each child or if the data for the four children are averaged for each period and then compared with the percentage retentions of the lactose period, the average increases in calcium retention during the lactose period are 35 and 42%, respectively, according to the two methods. These values check extremely well with the 39% increase over period VI observed for these four children and afford additional proof that lactose provided for a more efficient utilization of calcium by these children.

Although each child retained a greater quantity of calcium while ingesting lactose during period VIII, the response was far from uniform; the values ranged from 7 to 89%. The low values for subjects C and D (i.e., 7 and 10.2%, respectively) possibly are not truly representative of the influence which lactose can exert on the calcium metabolism of these two children. Subject C, at least, exhibited a 61% increase in per cent retention when he was given lactose in conjunction with a lower level of calcium intake, and subject D actually retained as much calcium during period VIII as he did during an earlier period when he received 1600 mg. calcium daily. The low value computed for the latter child is undoubtedly due to the fact that his control period followed the lactose period, and a portion of the large quantity of calcium which he stored then may have resulted, in part, from a persistence of the effect of lactose—which phenomenon occurred in all subjects but W—long after this sugar had been withdrawn. A possible explanation, likewise, may be found for subject W's apparently poor response to lactose during period VIII. Although during periods II, III, IV, and V, this child had consistently retained only about one-fifth of his calcium intake (to be exact, 20.7, 21.1, 20.6 and 20.4%, respectively), his per cent retention during the control period unaccountably jumped to 26.6%. If the latter value should represent the maximal extent to which this child could retain calcium, then it is obvious that an effect of lactose could not be demonstrated. However, this child did not respond to lactose during period X, and it is possible that

the value of 10.2% accorded to him for period VIII does represent the maximal effect which lactose would have on his calcium metabolism. On the other hand, the great increase in retention (89%) computed for subject Br may be too high, in view of the fact that his retention of calcium was unduly low during the control period (i.e., it was 16.2% of the intake in contrast to values of 17.4, 19.0 and 17.0, respectively, for periods II, III and IV).

This discussion of the possible factors responsible for the lack of consistency in the data does not alter the fact that each child did show some improvement in his ability to retain calcium during period VIII. It is significant also that these effects were superimposed on any which might have resulted from the administration of liberal quantities of vitamin D. One can conclude, therefore, that the calcium utilization of man, like that of other species, can be influenced by the ingestion of the di-saccharide, lactose.

The metabolism data do not reveal the mechanism by which lactose aids the body in retaining calcium. However, it seems significant that, coincident with the ingestion of lactose there was a statistically significant trend toward a decrease in the calcium lost by way of the kidney. That this decrease in urinary calcium was mainly responsible for the increase in calcium retention is evident when the data for both are computed on the basis of calcium intake; the average percentage decrease in urinary calcium approximates the average percentage increase in calcium retention (subject W, during period X, is the only child who does not show this relationship). However, this is the only study in which, to date, a significant decrease in urinary calcium as a result of lactose feeding has been reported. In fact, Gross ('27) found an increase in the urinary calcium excreted by normal dogs; Outhouse, Smith and Twomey ('38), on the other hand, found no change in the urinary calcium of rats, nor did Robinson, Huffman and Mason ('29) who worked with calves. If subsequent lactose studies on children should confirm these data on pathways of calcium excretion, one might be justified in concluding that lactose exerts its effect, not by rendering the dietary calcium more

absorbable in the intestinal tract, but by making more utilizable that calcium which had found its way into the blood stream. Such a conclusion would seem more tenable if one accepts the contention of Kinsman et al. ('39) that growing children do not excrete endogenous calcium when ingesting inadequate amounts of calcium; if the urinary calcium is not of endogenous origin, it must, then, be unutilized absorbed calcium. It seems logical to believe that the ingestion of lactose, and the possible conversion of a portion of it to an organic acid of a type which could bring about a more complete ionization of the calcium in the blood stream (Greenwald, '38), could make some of this "wasted" calcium available for calcification purposes. On the other hand, the conventional explanation (advanced in 1923 by Dragstedt and Peacock) for the effect of lactose is that calcium absorption from the intestines is increased; in order to fit this theory to the data observed in this study, it would be necessary to assume a decrease in the permeability of the kidney to calcium with a consequent rerouting of this calcium to the intestines where it is excreted.

SUMMARY

The ability of lactose to influence the calcium retention of five little boys ranging in age from 5 to 7 years was investigated. Throughout the 13 to 16 weeks of the experiment, the children were fed a basal dietary to which $CaHPO_4$ was added in order to raise the level of calcium intake to approximately 500 mg. daily; vitamin D also was fed. For 6 to 9 consecutive weeks an additional supplement of lactose (36 gm. daily) was given. Calcium balances were determined, and the increase in percentage retention of calcium during the lactose period was used as the criterion of the effect of lactose on calcium utilization. The values for per cent retention during the non-lactose and lactose periods, respectively, were 26.6 and 29.3 for W, 26.5 and 29.4 for D, 20.1 and 21.5 for C, 16.2 and 30.6 for Br, and 14.0 and 21.1 for Jw. Obviously, the values for the lactose period were higher in every case; they constituted gains over the retentions of the non-lactose period of 10.2, 10.9, 7.0, 88.9 and 50.7% with an average of 33.5.

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THE EFFECT OF PYRAZINE ACIDS AND QUINOLINIC ACID ON THE V-FACTOR[1] CONTENT OF HUMAN BLOOD AND UPON CANINE BLACKTONGUE

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FOUR FIGURES

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In this paper observations are recorded which bear on the question whether nicotinic acid can be replaced in some of its functions by pyrazine monocarboxylic acid, pyrazine 2, 3-dicarboxylic acid, or quinolinic acid. It has been reported that quinolinic acid does not cure canine blacktongue (Woolley et al., '38), and that it does cure pellagra (Vilter and Spies, '39). This distinction is important, if confirmed, as it would be the first demonstration that the nutritional deficiencies underlying the two diseases are not identical, excepting the Chittenden-Underhill syndrome of blacktongue due to vitamin A deficiency (Chittenden and Underhill, '17; Underhill and Mendel, '28; Smith, Persons and Harvey, '37). It has also been reported that pyrazine monocarboxylic acid and pyrazine 2, 3-dicarboxylic acid cure promptly the glossitis of pellagra and that the mono-acid causes an increase in the V-factor content of human blood corpuscles and urine (Bills, McDonald and Spies, '39), as nicotinic acid does (Vilter, Vilter and Spies, '39; Kohn, '38); the pyrazine compounds did not produce in humans the marked vasodilator symptoms which often follow the administration of nicotinic acid.

[1] V-factor = Coenzymes I and II, and possibly unknown related compounds.

EXPERIMENTAL

Quinoxaline was synthesized by the method of Hinsberg (1887) from glyoxal and *o*-phenylene-diamine. Alkaline permanganate oxidation yielded pyrazine 2, 3-dicarboxylic acid (Gabriel and Sonn, '07), M.P. 186° C. Decarboxylation occurred on refluxing the latter for 15 minutes in glacial acetic acid, with an almost theoretical yield of pyrazine monocarboxylic acid, M.P. 221° C. There was no depression of the melting points of these compounds when mixed with samples supplied by Dr. C. E. Bills. Commercial quinolinic acid was used, and in view of the recent report that it may contain a considerable amount of nicotinic acid (Koser et al., '40) our sample was tested by treating an aqueous solution with cyanogen bromide and metol (Bandier and Hald, '39) and analyzing the absorption spectrum of the resulting colored solution. It contained less than 0.03% of nicotinic acid. By the same method the samples of the pyrazine acids were shown to be entirely free from nicotinic acid.

Effect on V-factor of human blood in vitro. It has been established that when defibrinated normal human blood is incubated under sterile conditions with nicotinic acid the V-factor content of the blood is increased (Kohn and Klein, '39). We have conducted similar experiments to find whether quinolinic acid and the pyrazine acids would also lead to the synthesis of V-factor. The data are collected in table 1 and it will be seen that neither quinolinic acid nor the two pyrazine acids stimulated the synthesis of V-factor. There is one low aberrant figure among those for nicotinic acid, and one aberrant high figure for pyrazine 2, 3-dicarboxylic acid, but the error of the method warrants disregard of these. (It has been shown that in comparative readings as used here, one assay in three may be expected to err by ± 7% and one in twenty by ± 16%, Kohn et al., '39.)

Synthesis of V-factor by blood in vivo. Healthy adult subjects on normal diets were observed. On the first and third experimental days, venous blood was assayed for V-factor (Kohn and Bernheim, '39) to establish a baseline value for

each individual. Drug administration was begun on the third day, after the blood sample had been drawn. The compounds were dissolved in water, approximately neutralized, and were given by mouth three times a day for 3 days, each dose being stoichiometrically equivalent to 5 mg. of nicotinic acid per kilogram body weight. Additional blood samples for assay were drawn after the fifth and ninth doses.

TABLE 1

Increase of V-factor content of human blood cells during incubation with various compounds for 18 hrs. at 34°C.

COMPOUND	CONCENTRATION (MILLIMOLS PER LITER)	PERCENTAGE INCREASE (EACH FIGURE FROM A SEPARATE TRIAL)
Nicotinic acid	0.40	31
	0.81	43, 49, 13, 30
	2.43	43, 41
Pyrazine mono carboxylic acid	0.81	4, 0
	2.43	6, 5, 0
	7.29	4
Pyrazine di-carboxylic acid	0.40	−2
	0.81	2, 12
	2.43	7
	4.1	3
	12.3	2
Quinolinic acid	0.81	0
	2.43	3, −5

For each datum the result was expressed as the ratio of V-factor concentration after incubation in the experimental tube to the concentration in an otherwise similar tube from which the test compound was omitted. Thus for 0.40 millimolar nicotinic acid the ratio was 1.31, therefore the V-factor concentration had increased 31% over the control tube.

The results are plotted in figure 1, and show that none of the test substances caused a significant rise in the V-factor content of the blood cells. After a lapse of 4 weeks the effect of nicotinic acid was determined on two of the subjects, and a third acted as control without treatment. Four doses of nicotinic acid were given to each subject over a period of 24 hours, each dose being 5 mg. per kilogram body weight. Blood samples drawn before and after the dosing period showed ele-

vation of the blood V-factor of the subjects who received nicotinic acid, comparable with similar elevation reported previously (Kohn, '38).

One of the subjects (P.H.) suffered a severe flushing reaction after taking pyrazine monocarboxylic acid, and another (H.I.K.) experienced "prickly heat" of the skin following one dose of pyrazine 2, 3-dicarboxylic acid.

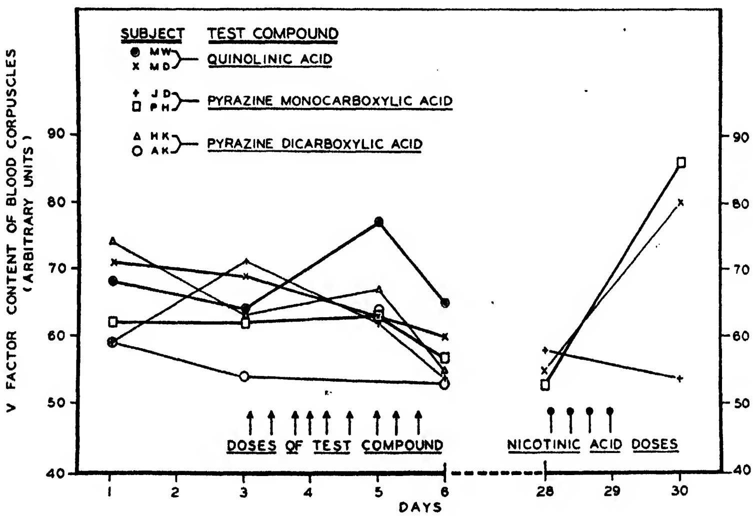


Fig. 1 The effect of oral ingestion of quinolinic acid, pyrazine monocarboxylic acid and pyrazine 2, 3-dicarboxylic acid on the V-factor content of human blood corpuscles. Each dose given was the molecular equivalent of 5 mg. of nicotinic acid per kilogram body weight of the subject. No significant increase occurred with any of the compounds; the increase for MW after treatment on the fifth day may be regarded as within the error of the method as it was not sustained; it compares with the apparent increase for JD before dosing was begun. In the later experimental period nicotinic acid was taken by MD and PH while JD served as control not dosed with nicotinic acid.

Effect in canine blacktongue. Each of the three acids was tested for blacktongue curative activity, by the standardized procedure previously described (Dann and Subbarow, '38), modified by giving each dog 2 mg. riboflavin and 2 mg. thiamin weekly in addition to the basal diet. The results of the curative

tests were variable and uncertain. With each acid, some of the dogs continued to decline after treatment and soon died of blacktongue, while others showed a rapid remission of mouth symptoms, return of appetite, and partial restoration of weight. In the latter animals the appetite generally remained good for some time, but the weight usually began to fall again a few days after treatment ceased, and the mouth signs returned sooner than after a curative treatment with nicotinic acid. No animal was fully cured, as judged by all of the following criteria—amount of weight restored; length of period after treatment until weight declined again; length of periods of restitution of appetite and freedom of mouth signs.

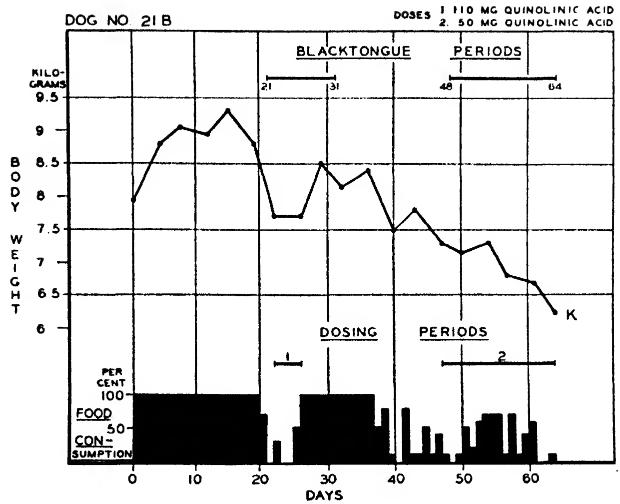
Four dogs were treated with quinolinic acid in doses of 3.33 to 13.75 mg. (In the curative tests, the dose signifies the weight of substance given per kilogram body weight each day for 5 days. Thus a 10-kg. dog on a dose of 3.33 mg. would receive in all 166.5 mg. during the 5-day dosing period. Under our conditions a dose of 2 mg. nicotinic acid causes complete cure.) The best response was seen in dog 21, receiving 13.75 mg.; its weight prior to the dosing period had decreased from 9.3 to 7.7 kg. Following the treatment it increased to 8.5 kg. 2 days after the last dose was given and then began to fall again immediately. The dog was not cured, although it received a dose stoichiometrically five times greater than the dose of nicotinic acid which gives a maximum response.

Seven dogs received pyrazine monocarboxylic acid in doses of 2 to 10.25 mg. None of the animals was completely cured, and there was no regular gradation of increasing response to higher doses. One of the best responses was shown by dog 17, given a dose of 3 mg. in its first attack of blacktongue. Its weight fell from 11.5 to 9.5 kg. before treatment and recovered rapidly to 10.5 and more slowly to 10.7 kg. on the eleventh day after treatment was completed. Then it began to fall rapidly and on the twentieth day after treatment was 8.6 kg. Normal appetite was restored for 20 days and then completely disappeared; the mouth signs returned on the eighteenth day after treatment.

Five dogs received pyrazine 2, 3-dicarboxylic acid in doses of 7.6 mg. to 13.6 mg. (Stoichiometrically 13.6 mg. of the di-acid are equivalent to 10 mg. nicotinic acid.) Only one of these dogs showed any weight restoration at all, although three of them showed some remission of mouth signs. The best response was given by dog 10, receiving 13.6 mg. From a weight of 9.75 kg. it had decreased to 8.8 kg. before dosing commenced, and further declined to 8.15 kg. but rose to 8.75 kg. on the fifth day after the last dose was given and then immediately began to decrease again. Appetite returned to normal but the mouth symptoms never disappeared or showed remission, and 10 days after the last dose treatment with 2 mg. nicotinic acid was begun and gave a complete cure.

These results indicated that none of the three acids tested was as potent a specific in blacktongue as nicotinic acid, although considerable improvement in mouth condition and appetite was produced in some of the dogs. In order to test more rigorously whether any of these compounds can replace nicotinic acid completely if given in sufficient quantity, preventive trials were carried out.

In the preventive tests, dogs were selected which were either still normal after only a few days on the deficient regimen or which showed only slight signs of the onset of blacktongue. A dose of test substance was given subcutaneously each day, until the observation period was terminated. The results obtained on some of the individual dogs are shown in figures 2 to 4, and a summary of the results follows: Five dogs were treated daily with quinolinic acid at levels of from 1 mg. to 20 mg. respectively per kilogram. Those receiving the lower doses declined and were killed for tissue examination when mortally sick with blacktongue. The dog receiving 20 mg. per kilogram was maintained in health, as judged by weight, mouth condition and appearance, for 156 days, after which it was killed (fig. 2, dog 10B). Four dogs were treated daily with pyrazine monocarboxylic acid at levels from 1 to 10 mg. per kilogram; these all declined and were killed for examination. Three dogs were treated with pyrazine 2, 3-dicarboxylic acid



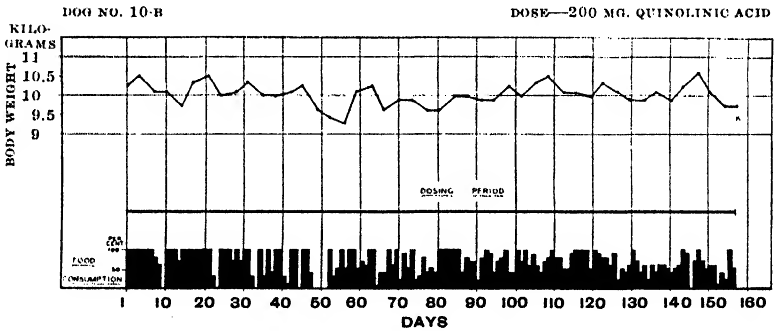
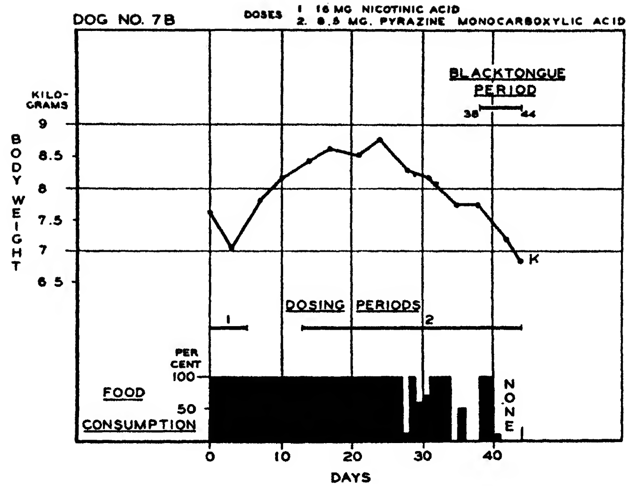


Fig. 2 Effect of quinolinic acid in blacktongue. Dog no. 21B increased in weight for 2 weeks on the basal diet, then developed blacktongue and showed a partial cure after receiving five daily doses each of 11.6 mg. quinolinic acid per kilogram (equivalent to 8.5 mg. nicotinic acid). The dose is calculated on the maximum weight of the dog in health. The mouth signs cleared up, but weight was only partly restored and the appetite returned for less than 10 days. Before the mouth symptoms returned preventive dosing was begun with 50 mg. quinolinic acid (equivalent to 4 mg. nicotinic acid) per kilogram daily. Blacktongue reappeared and the dog declined continuously until sacrificed. Dog 10B was normal at the beginning of the period shown and was given 200 mg. of quinolinic acid daily. Normal health and weight were maintained for 156 days as shown.

Note: "Blacktongue periods" in all figures refer to periods during which the characteristic mouth lesions were present. Food consumption indicates the percentage eaten of a standard weighed portion of the basal diet presented fresh daily to each dog.



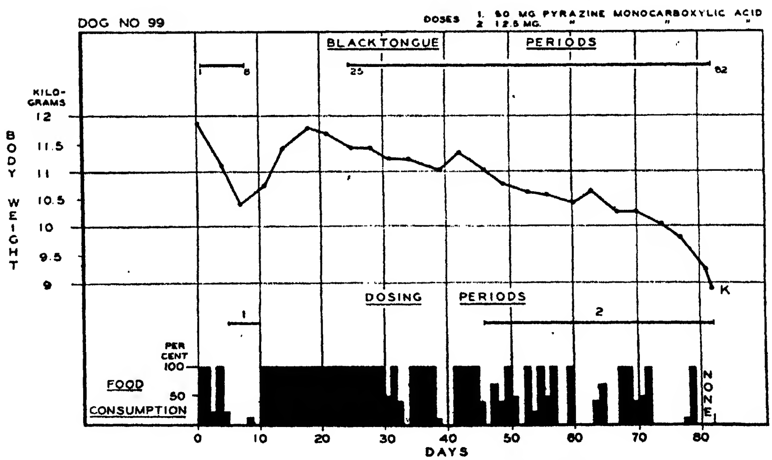


Fig. 3 Effect of pyrazine monocarboxylic acid in blacktongue. Dog 7B did not have blacktongue at the beginning of the period shown but was rapidly declining following an ineffective curative test which restored a normal mouth and appetite but did not prevent continued loss of weight. Five daily doses of 2 mg. nicotinic acid per kilogram caused full recovery. While the dog was quite healthy preventive treatment was begun with 1 mg. pyrazine monocarboxylic acid per kilogram daily, but the animal declined into mortal sickness as rapidly as though no treatment had been given.

Dog 99 first received a standard 5-day curative test and showed recovery for a limited period. The mouth signs soon returned, appetite then decreased, but only a slow loss of weight occurred. Treatment with 1 mg. per kilogram daily did not arrest the decline.

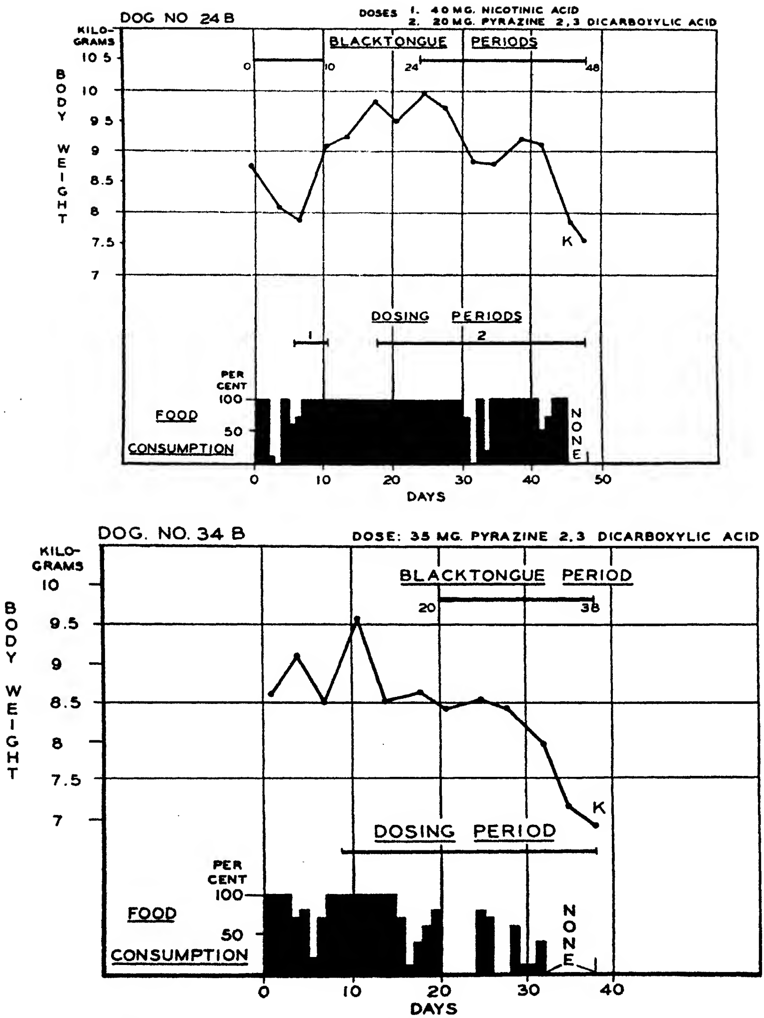


Fig. 4 Effect of pyrazine 2, 3-dicarboxylic acid in blacktongue. At the beginning of the period shown dog 24B was acutely sick following a curative test with an ineffective supplement; it was therefore given the large dose of 4 mg. nicotinic acid per kilogram daily for 5 days. When fully recovered from the attack it was given preventive treatment with 2 mg. pyrazine dicarboxylic acid daily, without retarding the reappearance of blacktongue.

Dog 34B was given the basal diet for the first time and while still healthy on the eighth day preventive treatment was begun with 4 mg. per kilogram daily. This did not give protection from blacktongue.

in doses of from 2 to 4 mg. per kilogram; and these also became sick and were killed when it appeared likely that they could not survive another night. Every dog except 10B showed marked signs of blacktongue before it was killed.

DISCUSSION

Our observations suggest that neither quinolinic acid nor the pyrazine acids raise the blood cell V-factor content when given orally to humans in doses more than twice as great as the amount of nicotinic acid causing at least a 50% increase. Similarly none of the three acids when added to defibrinated blood in vitro caused like nicotinic acid a marked synthesis of V-factor.

The disagreement between our results and those of Vilter and Spies ('39) and Bills et al. ('39) may be due to their use of H. influenzae instead of H. parainfluenzae, used by Lwoff and Lwoff ('37) in their identification of V-factor and employed in all our experiments. Also our subjects were all in normal health whereas they reported observations on pellagrins only; but this seems unlikely to be the cause of the difference as pellagrins and normal humans both show a similar increase in blood cell V-factor following the administration of nicotinic acid (Kohn, '38). A third variation in procedure is in the method of measuring the bacterial growth, which we estimated by photoelectric densitometry.

The experiments with dogs showed that in blacktongue none of the three acids exerted more than a fraction of the activity of nicotinic acid. In none of the curative tests was there a complete recovery although the stoichiometric ratio (largest dose of acid tested ÷ minimum dose of nicotinic acid insuring complete recovery) was 5 for quinolinic acid, and 10 for each of the pyrazine acids.

For an evaluation of the preventive tests it is necessary to adopt a figure for the level of nicotinic acid which will prevent blacktongue and maintain health and weight. From the "standard curves" of Margolis et al. ('38) it is seen that 5 mg. of nicotinic acid per kilogram body weight, spread over 10 days,

will cure a dog of blacktongue and maintain it for 35 days until blacktongue reappears. This gives a figure of $\frac{5}{35} = 0.14$ mg. nicotinic acid daily which is probably somewhat higher than that needed to maintain a dog which is healthy at the beginning of the test period. Again, Birch ('39) has reported that 0.084 mg. per kilogram daily did not afford protection from blacktongue, but 0.13 mg. daily gave protection and permitted slow growth. We shall therefore take 0.15 mg. per kilogram per day as the preventive dose of nicotinic acid. Under our conditions a dose of quinolinic acid stoichiometrically twenty-five times greater was ineffective in preventing blacktongue; but a hundredfold greater dose gave complete protection for 156 days. A sixty-sixfold greater dose of pyrazine monocarboxylic acid and a twentyfold greater dose of pyrazine dicarboxylic acid (in terms of gram-molecular quantities) failed to prevent the appearance of blacktongue.

Our results with quinolinic acid do not conflict with the observation of Woolley et al. ('38) that one dog weighing 5.6 kg. was not cured by a single dose of 150 mg. On the other hand they do conflict with the results obtained on human pellagra by Vilter and Spies. At approximately the same level of dosage Vilter and Spies ('39) found quinolinic acid to be as effective as nicotinic acid (Spies et al., '38). This conflict might indicate a species difference, contrary to our basic assumption that the deficiencies underlying the two diseases are identical. The close correspondence between dietary supplements effective in the treatment of pellagra and blacktongue, both qualitatively (Sebrell, '34) and quantitatively (Ruffin and Smith, '39) argues against this. Since Koser et al. ('40) suggested that commercial quinolinic acid may be contaminated with sufficient nicotinic acid to produce the cures seen by Vilter and Spies, and we now find quinolinic acid much less effective in blacktongue, a re-examination of its activity in pellagra is suggested. It is possible that moderate doses may produce temporary remission of the mouth signs without curing the underlying deficiency, as they do in blacktongue.

The results with the pyrazine acids in blacktongue treatment also conflict with the observations of Bills et al., ('39) on pellagra.

SUMMARY

1. Quinolinic acid, pyrazine monocarboxylic acid or pyrazine 2, 3-dicarboxylic acid taken orally by humans do not cause a rise in blood cell V-factor content.

2. None of the three acids caused a synthesis of V-factor when incubated under sterile conditions with defibrinated human blood.

3. None of the three acids was effective in curative trials on blacktongue. In preventive trials either 5 mg. quinolinic acid per kilogram, 10 mg. pyrazine monocarboxylic acid per kilogram or 4 mg. pyrazine dicarboxylic acid per kilogram daily was insufficient to prevent blacktongue, but 20 mg. quinolinic acid daily was preventive. These figures contrast with 0.15 mg. nicotinic acid daily, which is sufficient to prevent blacktongue.

Our thanks are due to the John and Mary R. Markle Foundation for a grant in aid of this study; to Merck and Company for supplying the thiamin and riboflavin used; and to Dr. C. E. Bills of Mead Johnson and Company for supplying part of the pyrazine acids tested.

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THE LENGTH OF TIME REQUIRED FOR DEPLETION OF VITAMIN A RESERVES IN RANGE CATTLE[1]

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Although considerable information is available as to the time required for depletion of vitamin A reserves in laboratory animals and scattered items may be found for some other species, nutritional literature contains very limited information pertaining to the depletion time of different species under varied conditions. The data presented herein pertain to range cattle over a period of 5 years and are a part of the results of an investigation designed to determine the carotene requirement for fattening beef cattle.

REVIEW OF LITERATURE

The use of cottonseed and its by-products, cottonseed meal and cottonseed hulls, as cattle feeds in the southern states has provided a fertile field for study of vitamin A deficiency in the bovine. In many cases, cattle fattened on cottonseed meal and cottonseed hulls became dull, listless, went "off feed," scoured and died as a result of the ration. Prior to 1900, cattle feeders knew this abnormal result as "fat sick-

[1] This report is part of an investigation to determine the carotene requirement for fattening beef cattle which became cooperative with the United States Department of Agriculture, Bureau of Animal Industry in 1935. A part of the data in this paper were presented in a paper entitled "Carotene Requirements for Fattening Beef Cattle" by J. H. Jones, J. K. Riggs, G. S. Fraps. J. M. Jones, H. Schmidt, R. E. Dickson, Paul E. Howe and W. H. Black, which was published in the Proceedings of the American Society of Animal Production, 1938, pages 94–102.

ness'' and recognized that it was often accompanied by inflammation of the eye or total loss of sight.

Connell and Carson (1896) attempted to produce ''fat sickness'' experimentally in native 3- and 4-year-old steers by feeding cottonseed meal and cottonseed hulls, but were unable to do so in periods of 180 days. This was the first attempt to produce the condition experimentally, and the admitted failure of these workers to do so may probably be explained through the fact that the experimental animals were aged steers from the area near the Gulf Coast of Texas where the annual rainfall approximates 40 inches and the growing season is about 250 days in length. The importance of these factors will be brought out later in this paper.

The involvement of the eye in this condition was thus early recognized, and the work of Halverson and Sherwood ('30) later substantiated it. Guilbert and Hart ('35) and Dickson, Jones and Schmidt ('35) established the relationship of night blindness and vitamin A deficiency in cattle. Halverson and Sherwood ('30) and Dickson, Jones and Schmidt ('35) have shown conclusively that the major factor in so-called cottonseed meal poisoning in cattle is the vitamin A deficiency of rations which have commonly been compounded from cottonseed meal and hulls to the exclusion of other feeds.

Halverson and Sherwood reported the appearance of unusual conditions of eyes, eye lesions, and dullness of sight without lesions, in a large percentage of steers after 88 days of feeding in dry lot on a ration composed exclusively of cottonseed meal and hulls. No mention was made of feeding prior to the time the test was begun. They also found that dairy heifers about 1 year of age may be fed such a ration for 150 to 250 days before acute symptoms of deficiency occur.

Guilbert and Hart ('34) observed night blindness in two Shorthorn steers, 11 and 17 months of age at the beginning of the experiment, in 248 and 252 days, respectively, on a vitamin A deficient ration after removal from conditions which afforded excellent opportunity for storage of vitamin A.

Mead and Regan ('31) reported development of avitaminosis A in calves 1 to 3 months after the diet was changed from whole milk and grain to a concentrate mixture low in vitamin A. Likewise, Moore ('39) found that two calves raised to 90 days of age on whole milk and grain and then fed skim milk together with a low carotene grain ration became night blind at 128 and 153 days of age or after 38 and 63 days, respectively, on the deficient ration. Bechdel and Skaggs ('36) observed symptoms of vitamin A deficiency in twelve dairy calves approximately 6 months of age after 70 days of feeding on a ration containing 37.2% of cottonseed meal but no roughage. Bechdel and Ward ('36) produced symptoms of avitaminosis A in five of six calves ranging in age from birth to 8 weeks by feeding a concentrate mixture of cereal grains and oil meals with poor quality timothy hay for 11 to 24 weeks.

These data indicate not only the variability in onset of symptoms of deficiency in different individuals but also the effect of age and the importance of feed supply and its vitamin A potency prior to the time the animals are placed on vitamin A deficient diets. The purpose of this paper is to present data pertaining primarily to the effect of age and seasonal range conditions upon the length of time required for depletion of vitamin A reserves in feeder cattle and calves as ordinarily marketed from ranges in the western part of Texas. It should be of especial value because of the large number of animals used in the work.

EXPERIMENTAL

The general plan of these experiments was to deplete the animals of vitamin A reserves in the body by feeding them a ration containing practically no carotene until they became night blind and then adding to the ration definite quantities of carotene in alfalfa meal during fattening periods varying in length. This paper is concerned only with the time required for depletion; therefore the discussion will be limited to that phase of the work.

Criteria for determining depletion

Previous investigations with cattle at this station (Dickson, Jones and Schmidt, '35) as well as at the California Station (Guilbert and Hart, '35) have shown that night blindness is the first symptom of vitamin A deficiency ordinarily detectable. The night blind condition appeared to be of progressive nature in nearly all cases in this investigation and therefore was measured in terms of degrees as shown below:

0—normal, no indication of defective night vision;
1—cautious, but can see objects at least 5 feet away;
2—cautious, but can see objects at a distance of 2 feet to a few inches;
3—totally night blind, cannot see.

Two consecutive observations of first degree night blindness or other definite assurance of affected night vision or of vitamin A deficiency were considered evidence of depletion. The animals were tested for night blindness out-of-doors by driving them about the pens and through 8-foot alleys formed by two board fences in which were located various obstacles such as inverted V-type hog troughs and movable panel barriers of unpainted wood or painted white on one side and black on the other. Testing was done at night as soon as twilight had passed.

Diets used and method of feeding

The depletion diets fed the various groups of cattle are shown in table 1. Cottonseed hulls constituted the roughage for all groups except 6 and 8 which received sorghum fodder having a carotene content of approximately 4 μg. per gram. Group 7 also received sorghum fodder, which contained 2 parts per million of carotene, for the first 58 days and was then changed to cottonseed hulls. The grain fed to groups 5, 6, 7 and 8 consisted of ground milo heads ranging in carotene content from 0.3 to 0.5 of 1 part per million. The total quantities of carotene derived from these feeds undoubtedly supplied a considerable part of the daily needs of the animals thereby lengthening somewhat the time required for depletion as com-

pared to what it would have been had cottonseed hulls formed the sole roughage in every case; however, the data were not materially affected. All of the animals were fed twice daily as groups except those in group 5 which were individually fed.

TABLE 1

Percentage composition of depletion diets

Components of ration	GROUP NUMBER							
	1	2	3	4	5	6	7	8
Tankage		3	3	3	3			
Cottonseed meal	12	10	10	10	15	13	12.5	15
Sorghum grain					30	38	25.0	28
Sorghum fodder [1]						49		57
Cottonseed hulls	88	87	87	87	50		62.5	
Limestone flour [2]	None				1	Ad lib	Ad lib	Ad lib
Salt	Ad lib	Ad lib	Ad lib	Ad lib	1	Ad lib	Ad lib	Ad lib

[1] Group 7 was fed sorghum fodder for the first 58 days and cottonseed hulls thereafter.

[2] Groups 3 and 4 were fed limestone flour at the rate of 0.10 pound per head daily.

Animals used

Two hundred seventy head of cattle have been used during the 5 years of this study. They were grade Hereford steers and heifers taken directly from the S.M.S. Ranch in Dickens County, Texas, to dry lots at Substation no. 7, Spur. In each case they were taken directly off the range at the time of purchase. The calves as groups ranged in age from 3 to 8 months and in weight from 225 to 467 pounds. The yearlings [2] were approximately 16 months old and averaged 559 pounds in weight. Table 2 shows the time required for depletion and gives the description of each group.

DISCUSSION

Wide variations are evident in the time required for depletion of different individuals (table 2). This appears to be true particularly in the case of older animals. The time re-

[2] Animals under 10 months of age are usually considered calves; 10 to 18 months, yearlings; and 18 to 30 months, 2-year-olds.

TABLE 2

The length of time required for depletion of vitamin A reserves in cattle of various ages

	GROUP NUMBER							
	1	2	3	4	5	6	7	8
Year	1935–1936	1936–1937	1937–1938	1938–1939	1939–1940	1938–1939	1939–1940	1939
Animals used:								
Class	*Steer yearlings*	*Steer calves*	*Steer calves*	*Steer calves*	*Heifer calves*	*Steer calves*	*Steer calves*	*Steer calves*
Number	48	40	50	50	30	10	20	12
Age in months	16	6–8	6–8	6–8	4–6	4–6	3–5	3–5
Length of depletion period in days:								
45– 59	..	..	..	..	..	..	..	6
60– 74	..	..	..	..	..	..	11	6
75– 89	..	..	..	10	1	10	6	..
90–108	..	9	1	20	25	..	2	..
109–128	5	1	16	15	4	..	1	..
129–148	9	18	19	3	..	..	..	..
149–168	10	8	13	2	..	..	..	..
169–188	5	2	0	0	..	..	..	..
189–208	5	2	1	..	..	..	..	..
209–228	13	..	..	..	..	..	..	..
229–248	0	..	..	..	..	..	..	..
249–268	1	..	..	..	..	..	..	..
Average	178 ± 37	136 ± 25	138 ± 21	107 ± 21	98 ± 7	86 ± 5	79 ± 17	56 ± 6
Range	128–266	101–206	96–194	82–155	82–112	83–90	65–131	46–61

quired for the yearlings in 1935–1936 ranged from 128 to 266 days with an average of 178 and a standard deviation of 37. One steer remained on the deficient ration for 381 days without becoming completely night blind, but showed numerous other symptoms of deficiency including swelling of the joints. This suggests a possible difference between individuals in ability to utilize carotene or vitamin A for a given physiological function. In contrast, the widest variation found in calves used in the years 1936 to 1939 was from 101 to 206 days. These wide variations in time required to become night blind show that different individuals have different reserves of vitamin A in the body. The smaller degree of variability in the younger animals also tends to indicate smaller storage in their case.

In the work with fattening cattle, the time required for depletion is determined by the body reserves of vitamin A which are affected by (1) the age of the animals, and (2) the vitamin A potency of the range forage. The effect of age may readily be seen in table 2. Younger animals have smaller reserves because of a shorter period of feeding on grass.

Appreciable variations are evident in the length of time required to deplete calves of the same age classification from year to year. The condition of the grass or range is determined by the amount and distribution of rainfall. Fraps, Copeland and Treichler ('34) have found that the feed of cows must be high in vitamin A potency in order for the animals to continue to produce butterfat high in vitamin A potency. Silage and ordinary hays and fodders apparently will not supply enough vitamin A potency to maintain a high content of vitamin A in butterfat. Green growing pasture grasses appear to be needed to maintain the production of butterfat high in vitamin A. When the leaves of plants dry, become brown and die, the vitamin A value may be completely lost (Coward, '25). In regions of frequently occurring drouths this suggests a relationship between rainfall and the vitamin A potency of the range forage.

Although the total annual precipitation for the years 1935 to 1938 varied only 4.51 inches and the departure from the 28-year average of 21.03 inches was not great, there was considerable difference in the rainfall for the 4 months, July, August, September and October, just preceding the time when all of these cattle except group 8 were removed to dry lot. During the fall of 1935, 1936 and 1937 the range grass made a good growth and was green until frost, but in 1938 as well as in 1939, there was little rainfall after the middle of July, and the grass was mostly dry from that time until frost. The average time required for depletion of the calves was 136 days in 1936–1937 and 138 days in 1937–1938. In these 2 years the July–October rainfall was relatively high. In 1938, however, when the July–October precipitation totalled only 4.93 inches, as compared to 15.50 and 12.26 inches in the 2 previous years, the average time required for calves of the same age was only 107 days. Slightly younger animals, taken from the range in the fall of 1939 when the July–October precipitation was 6.15 inches, required an average of 98 and 79 days for depletion of the two respective groups. One hundred per cent of these animals were depleted at the end of 128 days.

All of the groups shown in table 2 except no. 8 were purchased in the fall. After those in group 6 became depleted in an average of 86 days, the question arose as to the time for depletion required for animals of the same age which had been on the range not only during the fall but also during the dry winter months. The twelve animals in group 8, obtained for follow-up investigation, were removed from the range on March 17th just before the grass began spring growth and were started immediately on a depletion ration. At the end of 61 days they were all night blind, the average time being 56 days and the range 46 to 61 days.

Forty heifer calves approximately 5 months old were taken from the range on October 25, 1939, and placed on a vitamin A deficient ration composed of tankage, cottonseed meal, ground

milo heads and cottonseed hulls. They were divided into two groups of thirty head and ten head, respectively. The group of thirty, shown in table 2, required an average of 98 days for depletion. In addition to the depletion ration the group of ten head was fed 1000 μg. of carotene per 100 pounds live weight daily in dehydrated alfalfa leaf meal from the time they were brought in. These calves required an average of 113 days for depletion or only 15 days longer than those which were fed the unsupplemented depletion ration.

These findings indicate that range animals may go for considerable periods in the feed lot without suffering from vitamin A deficiency, but it is evident that these periods are not long enough for fattening to a high degree which may require 200 to 240 days in the case of young animals. This must naturally be modified according to the degree of deficiency of the fattening rations and the conditions which prevailed on the range where the cattle grazed.

SUMMARY AND CONCLUSIONS

A table showing the depletion time for 260 range cattle ranging in age from 3 to 16 months is presented. The data bear out the statement of Bessey and Wolbach ('39) that the accumulation of vitamin A in the body tends to increase with age and is dependent on the character of the diet. In dry years when only limited amounts of green vegetation are available on the range the time required for vitamin A deficiency to occur is less than in years of more abundant rainfall. This fact as well as the age of the animals must be taken into consideration when cattle are fed in dry lot for considerable periods of time on rations which do not supply an abundance of carotene or vitamin A.

One thousand micrograms of carotene (fed in dehydrated alfalfa leaf meal) per 100 pounds live weight daily added to the depletion ration for calves lengthened the average time required for the occurrence of night blindness only 15 days.

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PLACENTAL AND MAMMARY TRANSFER OF VITAMIN E IN THE RAT [1]

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In a previous report (Mason and Bryan, '38) evidence was presented indicating that placental transfer of vitamin E in the rat is negligible, and that mammary transfer is decidedly limited. When suckling rats, denied access to any source of vitamin E other than that obtained through the placenta and mammary glands of mothers receiving approximately three times the minimal daily requirement of the vitamin, were subsequently reared on an E-deficient diet, testicular degeneration appeared in the males within 30 to 40 days after weaning (51 to 61 days of age); while female offspring, inseminated at an average age of 66 days, consistently resorbed during their first gestation period. Increasing the maternal intake of vitamin E to fifteen and to 400 times the minimal, prior to the tenth day of lactation, induced a delay of approximately 20 and 30 days, respectively, in the onset of testis changes but failed to prevent first pregnancy resorptions in the female offspring. Other observations, involving exchange of litters between mothers given excess vitamin E and those on the stock diet, indicated that the vitamin E storage responsible for the delayed sterility in male offspring was largely, if not entirely, due to mammary transfer of the vitamin.

[1] This investigation was aided by a grant to Vanderbilt University School of Medicine from the Division of Medical Sciences of the Rockefeller Foundation.

The studies reported in the present paper represent an attempt to increase still further the state of E-hypervitaminosis in the pregnant and lactating female rat, to discriminate more fully between placental and mammary transfer of vitamin E under such conditions, and to measure the extent of this transfer by direct and indirect methods.

EXPERIMENTAL

The procedure used was essentially the same as that adopted in the previous study (Mason and Bryan, '38), except for the increased maternal intake of vitamin E. Normal female rats were mated and immediately transferred to the high-E diet, containing 20% by weight of a concentrate prepared from wheat germ oil by molecular distillation.[2] The composition of this diet, and that of our vitamin E deficient diet, was as follows:

High-E diet		*E-deficient diet*	
Casein (unextracted)	22%	Casein (unextracted)	20%
Cornstarch	54	Corn starch	48
Vitamin E concentrate	20	Lard	18
Salts	4	Salts	4
Brewers' yeast	1 gm. daily	Brewers' yeast	8
Cod liver oil	6 dr. daily	Cod liver oil	2

By the use of non-scatter food cups and daily records of food consumption, the intake of the concentrate was measured and the number of mean fertility doses for adult female rats estimated on the basis of bio-assays simultaneously made on this material. The limited supply of the latter permitted the use of only four rats at this exceedingly high level of vitamin E intake. At the fourteenth day of the 21-day period of lactation the high-E diet was replaced by the E-deficient diet. It was estimated that the daily vitamin E intake of each rat during pregnancy and during the first 2 weeks of lactation, respectively, was at least 2000 and 3000 times the minimal daily requirement for female rats; the larger intake during lactation

[2] We are indebted to the Research Laboratories of General Mills, Inc., Minneapolis, Minn., for generous supplies of this concentrate.

being due to the increased food consumption characteristic of this period.

Differentiation between placental and mammary transfer was accomplished by exchange of litters simultaneously delivered by high-E rats and by stock rats of the breeding colony, such exchange being made immediately after parturition and before any lactation occurred. The lactating mothers in both groups were transferred to the E-deficient diet on the fourteenth day of lactation in order to prevent the suckling young from obtaining any additional vitamin E through voluntary consumption of the maternal diet. Thus, offspring born of high-E mothers and fostered by stock rats represented the placental-transfer group; those born of stock rats and suckled by high-E rats constituted the mammary-transfer group. Six males delivered and suckled by the same high-E mother, representing the combined effect of placental and mammary transfer, were so indistinguishable from those of the mammary-transfer group in subsequent responses that they have been included with the latter in the summarized data (table 1). Special precautions were taken to prevent cutaneous absorption of the vitamin by the suckling young through physical contact with the high-E diet. The twenty female and twenty-six male offspring were weaned at the twenty-first day and continued on the E-deficient diet.

RESULTS

Extent of placental and mammary transfer of vitamin E. The left testis and epididymis of each male rat were removed by operation after different periods of experimental feeding and at a time when the early stages of testicular degeneration were expected. The latter were frequently ascertained by examination of stained smears obtained by puncture of the testis with a small hypodermic needle prior to operation. The other testis and epididymis were removed at autopsy 5 to 20 days later. After a careful examination of representative histological sections from both organs the testes were classified according to the predominant stage, or stages, of degeneration

present, using the arbitrary divisions of the degenerative process described by Mason ('26). On the basis of the histopathology of the germinal epithelium, supplemented by observations on the relative number and distribution of sperm and sloughed germ cells in the ducts of the epididymis, it was possible to estimate within very close limits the time at which the earliest morphological changes made their appearance. The latter analysis was aided by an extensive series of observations which we have made to determine the usual time interval between different stages of the degenerative process which, in general, is completed within a period of 15 to 20 days in our standardized male rats.

The time of onset of testis injury in rats of the placental-transfer group was not significantly different from that in the standardized controls (table 1).[3] However, when compared with the latter, the rats of the mammary-transfer group exhibited an average delay of 47 days in the appearance of demonstrable testis injury, which may be compared to the delay of about 20 and 30 days previously observed (Mason and Bryan, '38) when the maternal intake of vitamin E was approximately fifteen and 400 times the minimal, respectively. It thus appears that a maternal intake of vitamin E approximately 2000 to 3000 times the minimal permitted no demonstrable transfer of vitamin E through the placenta but increased the mammary transfer considerably over that occurring at much lower levels of vitamin intake, when delay in onset of testis degeneration was used as a measure of vitamin E storage in the newly weaned offspring.

[3] In earlier studies (Mason and Bryan, '38) it was noted that testicular degeneration appeared within 30 to 40 days after weaning in offspring of breeding females given access to both the stock and E-deficient diets during the first 10 days of lactation and to the deficient diet alone thereafter. In order to avoid the late-weaning paralysis which appeared in about one-fifth of litters prepared in this manner, we have since routinely transferred mothers and their litters from the stock diet to the deficient diet at the fourteenth day of lactation. The progeny, continued on the E-deficient diet after weaning on the twenty-first day, are referred to as our "standardized" rats. These standardized males show testis injury within 35 to 50 days after weaning (average, 40 days).

The female offspring in each group were bred to normal males. Successful matings occurred within an average of 40 days after weaning, at which time testicular injury was appearing in males of the control and placental-transfer groups. Since the results of previous studies of a similar type indicated that complete resorption would occur in all instances, several rats in each group were given doses of a vitamin E

TABLE 1

Showing extent of vitamin E storage, due to placental and mammary transfer, in young rats reared from weaning upon an E-deficient diet

GROUP	DELIVERED BY MOTHER FED	NURSED BY MOTHER FED	MALES: Number of rats	MALES: Days on experiment at onset of testis degeneration[1]: Min.	Max.	Aver.	FEMALES: Vitamin E conc. fed on 4th day of pregnancy	FEMALES: Number of rats	Responses: Neg.	Pos.[2]
							mg.			
S (standardized controls)	Stock diet	Stock diet[3]	42	34	50	40	0	35	35	0
							10	15	15	0
							15	9	9	0
							20	31	15	16
P (placental transfer)	High-E diet	Stock diet[3]	11	38	47	42	0	6	6	0
							10	2	2	0
							20	3	2	1
M (mammary transfer)	Stock diet	High E-diet[3]	15	72	98	87	0	4	4	0
							10	3	0	3
							20	2	0	2

[1] Estimated on the basis of histological examination of testes and epididymides removed at operation and at autopsy after different periods on the E-deficient diet.

[2] Based on the presence of two or more living fetuses at autopsy on the sixteenth day of pregnancy.

[3] Replaced by vitamin E-deficient diet during last week of lactation.

concentrate representing (1) a subminimal dose of 10 mg. consistently giving negative results in bio-assay tests, and (2) a dose of 20 mg. giving a positive response in approximately 50% of tests (see group S; table 1). All rats were autopsied on the sixteenth day of pregnancy and the presence of two or more viable fetuses was taken as a criterion of positive response. The advantages of this method of evaluating responses

will be discussed in a later report. The data are summarized in table 1.

The untreated rats of all three groups resorbed during their first pregnancy. Rats of the placental-transfer group responded to the vitamin E dosage in essentially the same manner as did the controls, while those of the mammary-transfer group were definitely more sensitive. This indicates that they had received through the mammary secretions of the foster mother an appreciable amount of vitamin E which, however, was insufficient to permit completion of their first gestation. Although these deductions are based upon a very limited number of animals, they are in full accord with observations made on littermate brothers (table 1) and with the results of other experiments discussed below.

Direct measurement of placental transfer. Female rats maintained on the stock diet relatively low in vitamin E, and others maintained on a diet of fresh raw wheat germ (supplemented with inorganic salts) for about 1 month prior to as well as during the period of gestation, were allowed to deliver on a coarse wire screen in order to permit the young to drop out of reach of the mother before they had an opportunity to suckle. In some instances, the rats were autopsied on the twenty-first day of pregnancy so as to obtain placentae and uteri as well as the fetuses for bio-assay tests. These tissues, and the newborn offspring, were frozen at 0°C. and fed, within about 10 days, to standardized females during early stages of their first pregnancy (table 2). As much as 200 gm. of newborn from stock rats gave only a subminimal response, characterized by the presence of a single well developed, but dead, fetus at the sixteenth day of pregnancy. On the basis of our experience with assay tests of this type it is estimated that approximately 250 gm. would have been required to give positive responses equivalent to those given by 50 gm. of newborn (ten young) delivered by high-E mothers. It is apparent that a demonstrable but definitely limited amount of vitamin E can pass the placental barrier. The low concentration of the vitamin in individual offspring of high-E mothers,

amounting to one-tenth the prophylactic dose for adult females, explains our failure to detect an appreciable storage of E in the placental-transfer group of rats (table 1) by the indirect method of approach. Other bio-assay tests of placentae and uteri obtained from high-E rats autopsied just prior to, or immediately after, delivery (table 2) indicate that the concentration of vitamin E on the maternal side of the placental barrier was more than five times that present in the fetuses or newborn.

Indirect measurement of mammary transfer. In order to determine the storage of vitamin E which must be present at weaning to permit a delay in the onset of testis changes comparable to that observed in the mammary-transfer experiment, two groups of twenty-one and seven standardized male rats were fed, during the first 3 days after weaning, varying doses of two different concentrates of wheat germ oil representing approximately 1, 2, 4, 8, 12 and 16 times the mean fertility dose.[4] These preparations were administered as ether solutions evaporated on a casein-dextrin mixture and fed as single doses after a preliminary starvation period of 6 to 10 hours. The onset of testicular injury was determined according to the procedure outlined in an earlier paragraph. From the data presented in table 3, it will be noted that the amount of concentrate X required to effect a delay in onset of testis injury

[4] The term "mean fertility dose," first proposed by Bacharach ('38), denotes the dose capable of giving a positive response in 50% of assay tests. It must be kept in mind that the response given at critical levels of dosage may be appreciably modified by differences in the period over which the test dose is administered. At the beginning of these studies we were feeding the total dose as a single dose on the fourth day of the first pregnancy, fed on a portion of the diet. For sake of uniformity this procedure was followed in frequent assays of the concentrates during the 10-month period of their use in the studies reported, although we feel that direct oral administration of divided doses over 5 or 10 days is much to be preferred. In estimating the mean fertility doses fed, it has been necessary to allow for a slight decrease observed in the potency of the concentrates during the course of these experiments. A few differences exist between the figures presented here, in relation to the doses given and the delay of testis injury observed, and those cited in a preliminary report (Mason, '39). The latter estimates were based upon a combined evaluation of the two groups of males in table 3 and were made before the complete bio-assay and histological data had been obtained and analyzed.

TABLE 2

Bio-assay tests showing the vitamin E storage in the newborn offspring of rats fed the low-E stock diet, and in the newborn, suckling young (24 hours old), placenta, uterus and mammary gland of rats maintained on a diet of fresh raw wheat germ for 1 to 2 months

TISSUE FED		UTERUS AT 16TH DAY L. D. R.[1]	TISSUE FED		UTERUS AT 16TH DAY L. D. R.[1]	TISSUE FED		UTERUS AT 16TH DAY L. D. R.[1]	TISSUE FED		UTERUS AT 16TH DAY L. D. R.[1]
Amount in grams	Days of pregnancy fed		Amount in grams	Days of pregnancy fed		Amount in grams	Days of pregnancy fed		Amount in grams	Days of pregnancy fed	
Newborn of stock rats			Newborn of high-E rats			Newborn of high-E rats (suckled first 24 hours)			Mammary gland (at term)		
200	3–7	0–1–7	50	4–6	5–1–2 (4)	30	4–6	9–0–0 (8)	10	5–6	11–0–0 (10)
120	4–7	0–0–13	50	4–6	2[2]–1–6 (0)	20	4–6	10–0–0 (10)	5	5–6	12–0–0 (11)
100	4–7	0–0–8	40	4–6	0–0–9	20	4–6	10–0–1 (10)	3.5	5–6	1[2]–2–4 (0)
90	4–8	0–0–8	40	4–6	0–0–11	15	5–6	12–0–0 (12)	2	5–6	0–0–11
60	3–5	0–0–10	40	4–6	0–0–9	15	4–6	3[2]–3–5 (1)	2	5–6	0–0–4
50	3–6	0–0–5	20	4–7	0–0–4	Placenta (21-day preg.)			(24 to 48 hours after delivery)		
50	3–5	0–0–8	20	4–8	0–0–8	20	5–7	7–0–0 (7)	7.7	5–6	8–0–0 (8)
40	3–8	0–0–10	20	3–5	0–0–6	10	5–6	2[2]–3–3 (0)	3.5	5–6	9–0–0 (9)
30	2–5	0–0–10	15	3–5	0–0–5	6.5	5–6	0–0–6			
			15	5	0–0–10	Uterus (21- to 22-day preg.)					
			15	5	0–0–9	10	5–6	8–0–2 (8)			
			15	5	0–0–11	2.2	5–6	0–0–9			

[1] Laparotomy was performed on all rats at the sixteenth day of pregnancy, and the number of live fetuses, dead fetuses and resorption sites recorded. The figures in parentheses indicate the number of fetuses delivered at term.

[2] Some uncertainty existed concerning the viability of fetuses recorded as living at laparotomy.

TABLE 3

Showing effect of single doses of vitamin E concentrates when fed to male and female E-deficient rats immediately after weaning

CONCENTRATE		TESTICULAR DEGENERATION IN MALES						REPRODUCTION IN FEMALES	
Amount fed, mg.	Number of mean fertility doses	Left testis (operation)		Right testis (autopsy)		Days on experiment at onset of degeneration (estimated)	aver.	Days on experiment at time positive mating	Outcome of first pregnancy
		Days on experiment	Stage of degeneration	Days on experiment	Stage of degeneration				
Concentrate X [1]									
0	Controls	45	1	60	3–5	42		35	Resorption
		45	1–2	60	5	41	40.5	38	Resorption
		45	1–2	60	5	41		41	Resorption
		45	2–4	60	5	38		42	Resorption
10	1	45	N–1	60	4–5	43		42	Resorption
		45	1	60	4–5	42	46.7	43	Resorption
		60	1–2	75	5	55		50	Resorption
20	2	45	N	60	N	. . .		31	Resorption
		60	N	75	N–1	73		37	Resorption
		60	N–1	75	4–5	58	67.2	39	Resorption
		75	1–2	90	4–5	71		45	Resorption
		75	3–4	90	5	67		. .	
40	4	75	N	90	1–2	86		34	Resorption
		90	N	105	1	102		37	Resorption
		90	1	105	3–5	86	87.6	41	Resorption
		90	2–4	105	5	83		48	Resorption
		90	3–4	105	5	81		. .	
80	8	90	N	105	N	. . .		36	Resorption
		90	N	110	5	95		44	Resorption
120	12	90	N	120	3–5	108		49	Resorption
160	16	120	1	135	4–5	116		. .	
Concentrate Y [1]									
0	Controls	50	2–3	70	5	43		29	Resorption
		60	4–5	80	5	46	44.5	30	Resorption
		. . .	. . .	. . .	. .	. . .		37	Resorption
100	4	50	N	75	2–4	68		29	Resorption
		60	N	70	1–2	66	67	43 [2]	Resorption
		. . .	. .	. . .	. . .	. . .		48 [2]	Resorption
200	8	60	N	80	N–1	77	76	27 [2]	Resorption
		80	2	90	4–5	75		36	Resorption
		. . .	. .	. . .	. . .	. . .		36	Resorption
		. . .	. .	. . .	. . .	. . .		49 [2]	Resorption
		. . .	. .	. . .	. . .	. . .		50 [2]	Resorption
300	12	. . .	. .	. . .	. . .	. . .		30	Resorption
		. . .	. .	. . .	. . .	. . .		33	Resorption
400	16	. . .	. .	. . .	. . .	. . .		31	Resorption
		. . .	. .	. . .	. . .	. . .		33	Resorption

[1] Concentrate X was an extract prepared from wheat germ oil, furnished us through the kindness of Dr. H. A. Mattill of the Department of Chemistry, State University of Iowa, Iowa City. Concentrate Y was the same molecular distillate of wheat germ oil as used in the high-E diet.

[2] Rats which failed to give a positive response when fed one-half the mean fertility dose of concentrate Y on the fourth day of their first pregnancy.

comparable to that observed in rats of the mammary-transfer group (table 1) corresponded to four times the mean fertility dose. In other words, the individual rats of the latter group must have acquired during lactation an average storage of E equivalent to four mean fertility doses. This, in turn, represents a storage forty times greater per rat than that resulting from placental transfer. However, on the basis of weights of 5 gm. and 40 gm. for newborn and newly weaned rats, respectively, the actual concentration of vitamin E per gram of tissue would be in a ratio of 1 to 5.

We have calculated that the rats of the mammary-transfer group possessed a storage of vitamin E equivalent to about one five-hundredth of the total intake of the mother during lactation. Since the four high-E rats suckled an average of 6.25 progeny, it is apparent that the average litter received through the mammary secretions about one-eightieth of the maternal intake during lactation. It must be stated, however, that the maternal storage depots may have been fully saturated by the high intake of E during pregnancy. Furthermore, since we have no data on vitamin excretion in the high-E rats, one cannot state what proportion of the vitamin intake was actually absorbed and retained by the maternal tissues. In terms of mean fertility doses, concentrate Y proved decidedly less effective in male rats than did concentrate X, but the number of animals tested was too limited to warrant very definite conclusions.

In contrast to the clear-cut response given by males to vitamin E dosage at the end of weaning, thirty-three littermate sisters treated in like manner and mated after an average experimental period of 39 days consistently resorbed during their first pregnancy (table 3). In fact, five of these rats failed to give positive responses when fed subminimal doses of one of these concentrates on the fourth day of pregnancy. These observations first suggested that the young female rat absorbs and stores vitamin E less effectively than the male, or else dissipates an excess of E more rapidly during prepubertal growth.

In order to obtain more specific information on this question thirty-eight standardized females were fed (according to the method used in the preceding study) either three or six times the mean fertility dose of concentrate Y, administered in three consecutive daily doses at periods of 14 to 30 days after weaning. Since single doses of this same size, administered in the same manner but on the fourth day of pregnancy, had been shown to give positive responses in standardized females, it was felt that the possibilities of partial destruction or poor absorption of the vitamin would thus be

TABLE 4

Showing the effectiveness of six times (120 mg.) and three times (60 mg.) the mean fertility dose of vitamin E concentrate (Y) fed as single doses to standardized female rats at intervals prior to the time of positive mating

120 MG. DOSE				60 MG. DOSE			
Days fed prior to mating	Implantation sites at autopsy on the 16th day of pregnancy			Days fed prior to mating	Implantation sites at autopsy on the 16th day of pregnancy		
	L. D. R.[1]	L. D. R.	L. D. R.		L. D. R.	L. D. R.	L. D. R.
27–24	0–0–12	1–0–8	0–0–11	15	1–1–7		
23–20	0–0–11	0–0–11	9–0–0	14	0–0–10	5–2–4	
18	0–0–9	2–2–7	4–1–2	13	0–0–10	1–0–9	3–0–8
17	5–2–4	5–2–3		11	2–0–8	6–0–5	
15	2–0–1	3–0–6	10–0–0	10	8–1–3	9–0–1	
13	3–0–5	4–1–3		9	2–0–10	2–5–3	
12	3–0–6	5–0–6	10–0–0	8	11–0–0	8–1–2	
10	6–0–4	8–0–2		5	2–0–8	7–0–3	8–0–1

[1] L (live fetuses), D (dead fetuses) and R (resorption sites).

eliminated. By subsequent regulation of the time of mating in the dosed females it was possible to obtain a series in which the second day of dosage occurred at intervals of 5 to 27 days prior to the time of insemination. The data obtained (table 4) indicate that six and three times the mean fertility dose became ineffective when fed about 3 and 2 weeks, respectively, prior to the day of mating. These results suggest that the females used much of the vitamin E administered for metabolic activities other than those related to reproductive functions. This is in accord with the observation that 2 mg. oral doses of

this same concentrate, after losing two-fifths of its potency during a year's storage, represented close to the minimal daily requirement for preventing sterility in standardized rats of both sexes (Mason, '40).

Apparently the male and female rats utilize minute daily supplements of vitamin E with equal efficiency in the satisfaction of their reproductive needs, and there is reason to believe that their capacity to absorb and store vitamin E is essentially the same. The seemingly paradoxical nature of the response of the two sexes to vitamin E received through the mammary secretions (table 1) or administered shortly after weaning (table 3) can now be satisfactorily explained on the basis of certain qualitative differences in sex requirements for vitamin E. These differences are characterized by the fact that vitamin E becomes effective in males prior to the twenty-fifth day after weaning, if irreversible disturbances in the seminiferous epithelium are to be prevented, whereas the reproductive needs of the female rat become acute only after conception and implantation and, consequently, arise at any stage of the reproductive period (Mason, '40).

Direct measurement of mammary transfer. Bio-assay data presented in table 2 indicate that vitamin E storage in the progeny of high-E mothers increases more than threefold during the first 48 hours of lactation [5] and that the mammary gland tissue itself represents a concentrated source of vitamin E. In fact, no other tissue from rats maintained for a similar period on the raw wheat germ diet has shown as high a vitamin E potency (unpublished studies). These findings are in accord with observations relative to the extent, and indirect measurement, of mammary transfer discussed above. Measurement of mammary transfer by direct bio-assay of the entire carcasses of newly weaned rats was not attempted.

[5] No attempt was made to discriminate between the vitamin E actually stored by the tissues, and that in the undigested milk present in the stomach, of the suckling young.

DISCUSSION

In view of the indispensability of vitamin E for the normal growth and development of the fetus in the rat, it is surprising that the maximum storage which the latter acquires during intrauterine life is so limited. It must be kept in mind that the observations presented in this report have dealt primarily with the extent of vitamin E storage in newborn and newly weaned rats as a result of placental or mammary transfer of the vitamin, irrespective of that which may have been directly utilized for general metabolic processes in the developing fetus and in the suckling rat. This metabolic utilization, which cannot be measured experimentally, is probably not very great in view of what is known regarding the minimal daily requirements of rats during the post-weaning period (Mason, '40).

Studies relating to the distribution of carotene and of vitamin A in the human placenta as well as in the fetal tissues point to an exceedingly limited transfer of these substances through the placenta (Wendt, '36; Gaehtgens, '37, '37 a). Likewise, the limited transfer observed in rats and rabbits can be augmented but little by feeding large amounts of vitamin A during pregnancy (Dann, '32, '34). The same appears to be true of the placental transfer of vitamin D in the rat (McCollum et al., '27). The decidedly restricted transfer of vitamin E across the placental barrier might be construed as a design of nature to protect the fetus against an excess of this vitamin, or to conserve for use of the maternal organism any vitamin over and above that required by the fetus. However, vitamins A and D behave in like manner and there is no evidence that an excess of any of the fat-soluble vitamins is really deleterious to the fetus. Consequently, the limitations of placental transfer of vitamin E must be attributed to the physiological selectivity of the placental barrier which, insofar as the fat-soluble vitamins is concerned, may represent a function of their molecular size.

Our observations concerning the more effective, but still definitely limited, transfer of vitamin E through the mammary

gland is in close accord with what is known regarding the mammary transmission of vitamin A in the rat and in man (Dann, '32, '36) and of vitamin D in rats (McCollum et al., '27). Additional studies are necessary to determine whether the concentration of vitamin E is greater on one side of the mammary barrier than on the other.

The earlier observations of Evans and Burr ('27), and unpublished studies from this laboratory in which 20% of butter fat from cows on green pasture was required in diets otherwise deficient in vitamin E in order to confer fertility on rats reared on the diet from weaning, indicate that mammary transfer of vitamin E in cattle is very limited. Advantage has recently been taken of the low content of vitamin E in cows' milk for the production in dogs of a condition of muscular dystrophy attributed to an inadequacy of vitamin E (Anderson et al., '39). A satisfactory approach to the problem of experimental vitamin E deficiency in various mammalian forms which do not thrive for long periods on the purified and concentrated type of diet customarily used for rats and mice might be facilitated by rigid restriction of the maternal intake of vitamin E prior to the time of weaning, followed by the use of diets composed largely of milk from animals receiving a low intake of E.

The time of onset of testis degeneration has proved a very sensitive index of the extent of initial storage of vitamin E in newly weaned rats, and constitutes a useful criterion of the rapidity and degree of vitamin E depletion in any colony of rats being used for experimental E-deficiency. In addition to observations presented in this report, one of us (K.E.M.) has had an opportunity to test this criterion in another laboratory possessing a large colony of rats devoted to the bio-assay of vitamin E.[6] For the purposes of this test, one or more male rats were included in litter groups of newly weaned

[6] We are greatly indebted to Mr. A. L. Bacharach, Director of the Biochemical Division of the Glaxo Laboratories, Ltd., Greenford, Middlesex, England, for his kindness in placing the facilities of his laboratory at our disposal and for his generous cooperation in these studies.

females being placed on the E-deficient diet. Smears of the testis and epididymis were obtained from males autopsied at intervals after the sixtieth day of age. Differences of about 20 days in the onset of testis injury were readily detected by this method in two series of E-deficient rats which differed only in their vitamin E intake during lactation. No great experience is required to ascertain the onset of early stages of the degenerative process, characterized particularly by the appearance of numerous abnormal spermatozoa scattered individually or in clumps in different portions of the smear, without the necessity of examining histological sections of these organs.

In the early reports of Evans and Burr ('27) the condition of "initial fertility" observed in their E-deficient female rats, and noted by many other investigators since that time, was explained on the assumption that young animals are born with appreciable stores of vitamin E which, although slowly dissipated by the various metabolic processes during early growth and development, are supplemented by traces of E present in the purified diet to such an extent that a variable number of females are enabled to complete one or more gestations before sterility intervenes. The observations reported here, and elsewhere (Mason and Bryan, '38; Mason, '40), indicate that (1) the maximum result of combined placental and mammary transfer of vitamin E falls far short of conferring fertility on female rats deprived of any other source of the vitamin, (2) the usual components of E-deficient diets do not contain sufficient traces of E to augment appreciably the animal's stores of the vitamin, (3) the storage which newly weaned rats acquire through voluntary consumption of stock diets of variable E-content represents the major cause of "initial fertility" in rats reared on E-deficient diets.

SUMMARY AND CONCLUSIONS

1. Comparisons have been made between the placental and mammary transfer of vitamin E by stock rats receiving an adequate but low intake of this vitamin, and by rats given

approximately 2000 to 3000 times the minimal daily requirement of vitamin E throughout pregnancy and the first 2 weeks of lactation.

2. When measured by the delay in onset of testis degeneration in male offspring otherwise deprived of vitamin E, no placental transfer was demonstrable but mammary transfer was sufficient to cause an average delay of 47 days in the appearance of testicular injury. This may be compared with a delay of about 30 and 20 days previously observed when the maternal intake of E was approximately 400 and fifteen times the minimal, respectively. Indirectly measured, the maximum mammary-transfer storage in individual rats amounted to about four times the mean fertility dose for adult females.

3. Placental and mammary transfer of vitamin E to female offspring otherwise deprived of vitamin E failed to prevent resorptions during their first pregnancy. These somewhat paradoxical observations are discussed in relation to qualitative time differences in the vitamin E needs of the two sexes.

4. On the basis of direct bio-assay tests the newborn from high-E rats (fed fresh raw wheat germ for 1 to 2 months) contained approximately five times as much vitamin E as those from the stock rats, indicating a demonstrable but decidedly limited transfer of the vitamin across the placenta to the fetus amounting to about one-tenth the mean fertility dose in each newborn rat. The concentration of vitamin E in the placenta and uterus of high-E mothers was about five times greater than in the full term fetus or newborn, but considerably less than in the mammary gland. After 24 hours of lactation the vitamin content of the newborn increased more than threefold.

5. It is concluded that placental transmission of vitamin E in the rat is exceedingly limited, and that the vitamin is much more readily transferred through the mammary gland to the milk. These findings are discussed in relation to the experimental production of vitamin E deficiency and to the question of placental and mammary transmission of other fat-soluble vitamins.

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MULTIPLE DEFICIENCIES IN THE MODIFIED GOLDBERGER DIET AS DEMONSTRATED WITH CHICKS [1]

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TWO FIGURES

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In our earlier studies on the chick antidermatitis factor (Mickelsen, Waisman and Elvehjem, '38) numerous attempts were made to produce a nicotinic acid deficiency in chicks through the use of the modified Goldberger diet. We found that chicks on this ration grew very poorly but showed no external symptoms. When the ration was supplemented with various levels of nicotinic acid there was a variable response in growth, indicating that growth was limited mainly by factors other than nicotinic acid.

While our work was in progress, Helmer and Fouts ('38) and Margolis, Margolis and Smith ('39) demonstrated that the Goldberger diet was deficient in thiamin and riboflavin as well as in nicotinic acid. Helmer and Fouts ('38) did their work with rats while the latter workers demonstrated the deficiencies in dogs. Further work in our laboratory showed that the modified Goldberger diet was deficient in several of the factors required by the chick. In this paper we wish to report the results of a rather extensive investigation on this well-known diet using the chick as the experimental animal.

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EXPERIMENTAL

The basal ration used in these studies was the modified Goldberger diet which we have fed to dogs in the bio-assay of nicotinic acid (Waisman et al., '40). Its percentage composition is: ground yellow corn 71, purified casein 18, salts 4, cottonseed oil 5, cod liver oil 2. Day-old White Leghorn chicks were used throughout the experiments. They were weighed

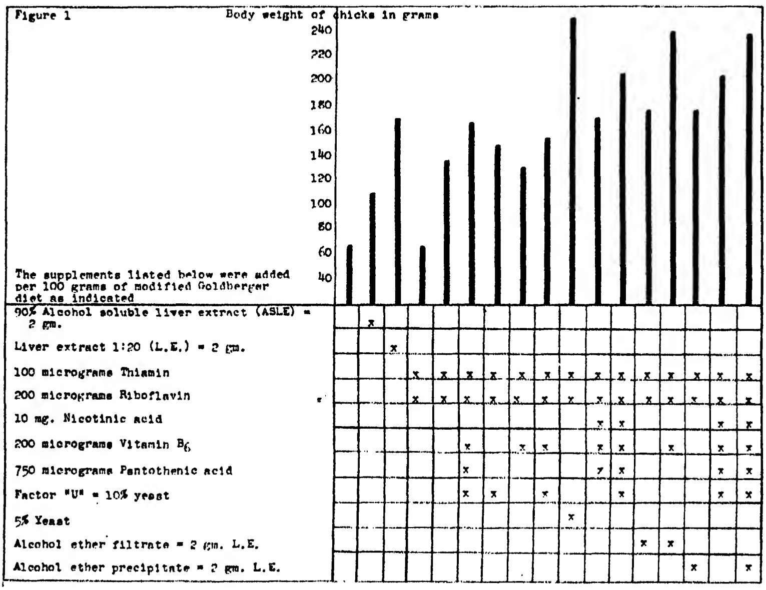

Fig. 1 Average growth responses at 5 weeks of chicks fed various supplements to the modified Goldberger diet.

weekly and carefully observed daily for indications of any symptoms. Water solutions of the supplements were dried on the basal ration. The chicks were given additional vitamins A and D in the form of halibut liver oil twice weekly by dropper.

RESULTS

The results of these experiments are given in very condensed form in figure 1. In many cases several different groups of

chicks were fed the same modifications of the basal ration but we are presenting only the average results. The modified Goldberger ration does not support growth during the assay period of 5 weeks. Mortality is high and the surviving chicks seldom double their original weight in 5 weeks. When the ration was supplemented with 2% of liver extract powder 1:20,[2] growth was fairly good. If the basal ration was supplemented with an alcohol soluble fraction of liver, made from the liver powder after the removal of the pernicious anemia factor, designated in the chart as ASLE, growth was improved over the basal but was far from optimum.

The addition of 100 μg. thiamin per 100 gm. of ration produced only a slight response but a marked growth response was obtained when 200 μg. of riboflavin was added. This result confirms the work of Helmer and Fouts ('38) and Margolis, Margolis and Smith ('39).

Our experiments indicate that some response was obtained in several groups receiving nicotinic acid. Although this ration produces black tongue in dogs and is low in nicotinic acid for this species, the results indicate that this ration does not lend itself for the demonstration that the chick requires nicotinic acid. Although the amounts of nicotinic acid added varied from 10 to 100 mg. per 100 gm. of ration the exact level of the vitamin which gave the best growth response in the chicks could not be determined. In several groups a definite growth gain was noted when the basal diet was supplemented with from 10 to 20 mg. of nicotinic acid per 100 gm. of ration. The growth stimulation by nicotinic acid was also noted when the ration was further supplemented by vitamin B_6, pantothenic acid, and factor U. Our data on a large number of chicks indicate that levels of nicotinic acid above 20–25 mg. per 100 gm. of ration were definitely toxic.

Vitamin B_6 has recently been shown to be essential for the growth of chicks (Carter and O'Brien, '39; Hegsted, Oleson,

[2] Supplies of liver extract were contributed by Wilson Laboratories, thiamin, nicotinic acid, vitamin B_6 and pantothenic acid by Merck and Company, yeast by Pabst Brewing Co., and haliver oil by Abbott Laboratories.

Elvehjem and Hart, '39, '40). When vitamin B_6 was added at a level of 200 μg. per 100 gm. of ration, growth was not improved over the groups receiving thiamin and riboflavin alone. However, when vitamin B_6 was added to the ration containing an alcohol ether filtrate fraction of liver extract (Elvehjem et al., '36), growth was increased above that of the modified Goldberger ration plus the alcohol ether filtrate fraction alone. This is an indication that the growth promoting effect of a particular supplement can be demonstrated only when the ration is more complete in the accessory food factors.

Pantothenic acid has been shown to be the chick antidermatitis factor by Woolley, Waisman and Elvehjem ('39) and by Jukes ('39). We have obtained data indicating that the basal modified Goldberger diet is low in this vitamin. Throughout several groups not receiving supplements of pantothenic acid, a number of chicks showed some dermatitis. Invariably no dermatitis was observed in those chicks which received a pantothenic acid concentrate. A number of groups fed a ration containing factor U (Stokstad and Manning, '38), vitamin B_6, riboflavin, and thiamin showed a definite growth increase when the ration was supplemented with pantothenic acid.

The addition of 5% yeast to the basal ration supplemented with thiamin and riboflavin, resulted in very good growth. The chicks weighed 265 gm. at the end of the 5 weeks' period. This average weight is superior to that of the chicks receiving the five known members of the vitamin B complex, i.e., thiamin, riboflavin, nicotinic acid, pantothenic acid, and vitamin B_6. This increased growth is adequate demonstration that yeast supplies a factor or factors other than the five mentioned above. From this observation we turned to the factor, required by chicks and found in yeast, called factor "U" by Stokstad and Manning ('38). This factor is concentrated by MeOH-H_2O extraction of yeast and subsequent absorption on fuller's earth and elution with NH_4OH or pyridine. This preparation would contain thiamin, riboflavin, vitamin B_6 and certain other factors besides factor U. When such a preparation was fed to the chicks, in amount equivalent to 10% yeast, together with thiamin and riboflavin, a definite response in growth was

obtained. If the factor U concentrate was fed with thiamin, riboflavin, nicotinic acid, vitamin B_6, and pantothenic acid, growth was definitely better than without factor U. However, the average weight of the birds did not reach that of the group on the 5% yeast. Another series indicated that factor U did not give its effect until vitamin B_6 was added. Vitamin B_6 alone did not improve growth nor did the addition of factor U, but when factor U was given together with vitamin B_6 the gain in weight was noteworthy.

Factors which were furnished by the yeast other than factor U may be unidentified members of the vitamin B complex. A definite stimulation to growth was obtained when a liver concentrate, the alcohol ether precipitate (Elvehjem et al., '36), was fed with the basal ration. This stimulation of growth by the alcohol ether precipitate was demonstrated in a number of ways. The response was noted when it was fed either alone or with any combination of the known members of the vitamin B complex. The growth response on alcohol ether precipitate together with any combination of the supplements showed that the factor (or factors) supplied by this concentrate is necessary for optimum growth of the chick fed the Goldberger diet. A definite response above the basal ration was obtained when the alcohol ether filtrate fraction from liver (Elvehjem et al., '36) was fed. This filtrate is known to carry riboflavin, nicotinic acid, and pantothenic acid, besides other factors. The increased growth on this liver fraction is explained by its content not only of known factors, but also by unknown factors, since the growth obtained with the liver fraction was greater than that with the known factors alone.

Interesting results were obtained when a natural foodstuff such as animal tissue was fed with the modified Goldberger ration. Figure 2 shows the growth obtained on various levels of these tissues. In order to conserve space only a few levels of meat are listed. The numbers following the meat samples in figure 2 refer to the sample numbers of the tissues used by us in previous studies. With as little as 1 or 2% pork or lamb liver, or beef kidney, growth was definitely superior to the basal. The growth on the 3% level of beef kidney was equal

to that obtained with 5% yeast. An increasing rate of growth was obtained with the increasing levels of the tissue. With 2 and 4% of veal liver, growth was very good. The addition of 5, 10 and 20% beef spleen to the basal ration resulted in progressively increased growth with increasing quantities of spleen. In one series the birds on the 10% spleen showed definite symptoms (curled toes) of riboflavin deficiency. According to the actual quantity of riboflavin supplied by this tissue (Mickelsen, Waisman and Elvehjem, '39) the riboflavin intake was borderline for the chick (Bethke et al., '37; Norris

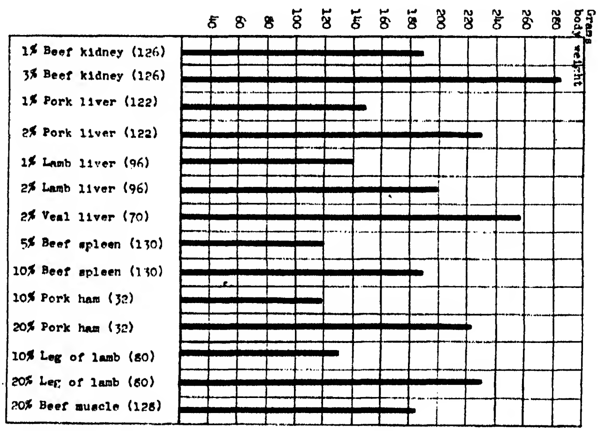

Fig. 2 Growth at 5 weeks of chicks fed various levels of animal tissues together with the modified Goldberger diet.

et al., '36). With the 20% level no symptoms of riboflavin deficiency were observed.

When pork ham was fed at 5 and 10% levels, sufficient thiamin was supplied, but the levels of riboflavin and pantothenic acid were too low to supply the requirements of the chick. It was to be expected therefore that the birds showed definite riboflavin symptoms as well as some typical dermatitis of the pantothenic acid deficiency. Another sample of pork ham was fed at 20 and 30% but the chick showed dermatitis especially at the lower levels. Veal muscle fed at 20 and 30% levels gave

complete protection against any symptoms. With 20 and 30% of beef muscle, growth was good.

The ability of organ tissue to supply most members of the vitamin B complex is greater than that of muscular tissue. This has been a consistent finding in previous studies and is again borne out by the experiments which serve to determine quantitatively the distribution of the members of the vitamin B complex in a particular foodstuff.

The growth studies on the modified Goldberger diet were augmented by observations of the blood picture of the chicks.[3] Hemoglobin, sedimentation rate, red cell count, and hematocrit were determined on most of the groups. We observed a definite lowering of the hemoglobin of the chicks on the basal diet. Hogan and Parrot ('39) have observed an anemia in chicks on a partially synthetic diet. The addition of riboflavin or any liver fraction to the basal diet resulted in increased hemoglobin level accompanied by the increased growth. It appeared that the poor growth was invariably accompanied by low hemoglobin. There was no change in the hematocrit, sedimentation rate, or red cell count when compared to chicks on a normal grain ration. A definite change in plasma color was noted when the group was supplemented with a liver fraction. Further work is being done to establish the cause of this plasma color.

SUMMARY

The limitations of the modified Goldberger ration have been determined with the aid of chicks and supplementation with various vitamins. Thiamin exerts no effect on growth, but riboflavin has been shown to give definite growth responses. Nicotinic acid was found to give variable responses in growth at levels below 20 mg. per 100 gm. of ration. Vitamin B_6 alone did not increase growth over the basal, but when added to a liver fraction which furnished additional factors, a definite response was obtained. The basal ration was found to be low in pantothenic acid and factor U. It has been demonstrated

[3] We are greatly indebted to Dr. Olaf Mickelsen and Mr. LaVell M. Henderson for their aid in some of the observations on the blood work.

that additional factors other than the known members of the vitamin B complex are necessary for optimum growth of chicks fed the modified Goldberger diet. Various levels of animal tissues have been found to give good growth when used as supplements to the basal diet. There is no significant change in the blood picture of the chicks other than a reduced hemoglobin content of the blood when on the basal diet.

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A QUANTITATIVE STUDY OF VITAMINS IN THE RUMEN CONTENTS OF SHEEP AND COWS FED VITAMIN-LOW DIETS

I. RIBOFLAVIN AND VITAMIN K

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FOUR FIGURES

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Data regarding the requirements of sheep and cattle for vitamins other than A and D are meager. Theiler, Green and Viljoen ('15) from their results in feeding a vitamin deficient diet to cattle over a period of a year concluded that "it may be that cattle are capable of synthesizing their own vitamins in virtue of the extensive bacterial flora of their intestinal tract." Scheunert and Schieblich ('23) showed that B. vulgatus, an obligate intestinal organism of hebivors is capable of synthesizing the anti-neuritic vitamin. The well-known work of Bechdel and his associates ('26, '27, '28) confirmed the opinion of Theiler, Green and Viljoen ('15) with regard to what was then known as the vitamin B complex. Largely on the basis of this work it has generally been assumed that the whole vitamin B complex may be synthesized in the rumen, and prior to preliminary reports (McElroy and Goss, '39, '40) of the present investigation little qualitative and no quantitative data had been reported in the light of the more recent knowledge of the multiple nature of the B vitamins.

In the Kenya Colony annual report ('34) it is suggested that "sweating sickness" of calves may be due to a deficiency of part of the vitamin B complex in the ration. Madsen, McCay

and Maynard ('35) have reported that sheep and goats maintained on a vitamin B complex deficient diet "require for growth some vitamin B factor found in yeast." Pearson, Schmidt and Mackey ('39) and Winegar, Pearson and Schmidt ('40) have presented evidence indicating that nicotinic acid is not a dietary essential for sheep.

Kramer and co-workers ('39) concluded that the ration of the cow does not have any significant effect on the riboflavin content of the milk.

Little is known regarding the dietary requirement of ruminants for vitamin K. Quick ('37) has shown that the vitamin K in alfalfa is not the factor responsible for its effectiveness in the treatment of sweet clover disease. Phillips and his associates ('38) have suggested that calves maintained on a ration low in vitamin K may suffer from a deficiency of this vitamin.

EXPERIMENTAL

Animals and their care. Experiments of essentially the same nature were conducted with sheep and cows. Four mature grade Dorset Horn ewes from the University flock were housed in a box stall with access to a small corral. No bedding was used and the concrete floor of the stall and corral was kept clean. Throughout most of the experimental period the ewes had a tendency to chew wood but no evidence of coprophagy was noted at any time.

The animals accepted the experimental ration readily and the change from the normal to the experimental ration was completed in 3 weeks. During the final week of the preliminary period only ¼ pound of alfalfa hay per head per day was fed in addition to the experimental ration. No attempt was made to keep individual feed consumption records. The four sheep ate from a common trough and within 30 minutes consumed an allowance of 2100 gm. given twice daily. The average initial weight of 137 pounds per ewe was maintained over the 30-day experimental period.

In the cow experiments three animals were used, but for reasons to be discussed in a later paper most of the vitamin

assays were conducted on rumen material collected from a single cow, a purebred Jersey no. 557, with a permanent fistula [1] into the rumen. The cows were housed in the same quarters as had previously been used for the sheep and were given the same care. The usual feed allowance was 4200 gm. per cow per day.

The fistulated cow no. 557 was maintained on the experimental diet over a period of almost 5 months. After she had been on the experimental ration for 80 days she gave birth to a normal 59 pound heifer calf. From birth to 43 days of age the calf fed solely on her dam's milk and remained in excellent condition gaining an average of 1.7 pounds per day.

Milk production was reasonably good considering the amount of feed the cow consumed (6 kg. per day during lactation). Since the calf was allowed to suckle at will it was not possible to keep accurate milk production records but it was estimated that a peak of between 25 and 30 pounds per day was reached during the second week of lactation. During the third and fourth weeks production fell to an estimated 20 pounds per day and was maintained at this level until the cow was taken off the experimental ration 62 days after parturition. Samples of the milk were saved for use in vitamin assays to be described in this and succeeding papers.

Sheep and cow ration. As previously described (McElroy and Goss, '39, '40) the experimental ration consisted of washed casein [2] 5%, washed sardine meal [3] 6.7, glucose [4] 11.0, corn

[1] For the preparation of the fistula and for assistance in the care of the animal following the operation we are indebted to Dr. G. H. Hart and Dr. H. H. Cole of this Division.

[2] Obtained from the Western Condensing Co., Petaluma, California. In the preparation of washed casein freshly precipitated sulfuric acid casein is washed nine times with five volumes of water brought to pH 4.5 with sulfuric acid. No acid is used in the tenth washing. Washing is effected by stirring the curd with the wash water for 1 hour, allowing it to settle and decanting. The final curd is allowed to drain and then dried in a current of warm air.

[3] Obtained from the Western Condensing Co. Commercial sardine meal is washed ten times with fifteen volumes of hot water (60°–70°C.) and dried in a vacuum oven or in a current of warm air.

[4] "Cerelose."

starch 10.6, mineral mixture 3 and dried plain sugar beet pulp 63.7%. During the later stages of the experiment the cows were given a supplement of 10 cc. per day of a halibut liver oil containing 50,000 U.S.P. units of vitamin A and 850 U.S.P. units of vitamin D per gram. To facilitate mixing of the diet a stock supply of a "concentrate" mixture was prepared. The percentage composition of this mixture was as follows: washed casein 15, washed sardine meal 20, glucose 33, and corn starch 32. The mineral mixture used was a modification of that described by Hubbell, Mendel and Wakeman ('37) and had the following composition (in grams):

$CaCO_3$	1500
KH_2PO_4	3000
NaCl	3000
Ferric citrate	200
$MgCO_3$	200
$CuSO_4 \cdot 5\,H_2O$	7.4
$K_2SO_4Al_2(SO_4)_3$	1.4
$MnSO_4 \cdot 7\,H_2O$	7.0
KI	0.66

The ration was prepared before each feeding by mixing 33.3 parts of the "concentrate" mixture, 3 parts of the mineral mixture and 63.7 parts of dried plain sugar beet pulp. This was moistened with two to three times its weight of water and was fed immediately in a clean trough.

Collection and treatment of rumen contents. The sheep were kept on the experimental ration for 30 days. It was assumed that after this length of time any residue of feed other than the experimental ration would have left the rumen. The sheep were then slaughtered 14 hours after the last feeding and the rumen and reticulum contents of each animal emptied into a separate stone crock. Enough 95% ethanol was added to bring the concentration of alcohol in the mixture to about 50%. This material was then stored in the cold and subsequently dried in glass dishes before a current of air at room temperature. The drying process required from 48 to 72 hours. When dry, the material was finely ground in a Wiley mill and stored in closed containers at room temperature until used in assay

diets. In all assays of sheep rumen and reticulum contents composite samples of equal weights of material from each of the four sheep were used.

The first sample of rumen contents was removed from fistulated cow no. 557 after she had been on the experimental diet for 43 days. During the following $3\frac{1}{2}$ months eleven additional samples of between 25 and 35 pounds wet weight were taken at intervals varying from 4 to 16 days. Samples were siphoned out through a $\frac{3}{4}$ inch rubber tube into stone jars. The rumen material was relatively liquid so that little difficulty was experienced in maintaining the siphon, and since care was taken to move the tube to different regions and levels of the rumen during the process of drawing the sample it is believed that this method permitted the collection of a representative sample of the rumen contents.

The sheep rumen and reticulum contents described above were taken in the summer when the atmospheric temperature was high and the humidity low so that rapid drying was obtained at room temperature, but due to the lower temperature and higher humidity prevailing while the cow rumen samples were being taken this method of drying could not be used for the cow samples. They were therefore dried before a current of air in an electrically heated oven at a temperature of 40° to 50°C. The first two samples were dried immediately after the addition of enough 95% ethanol to make the concentration of alcohol approximately 30%. The succeeding ten samples were first treated with alcohol and then with enough 36% HCl to lower the pH to 4.5 to 5.0 before being dried. In a chick assay described below it was found that a composite mixture of samples 3 and 4 which were acidified before drying contained about twice as much riboflavin as a composite mixture of samples 1 and 2 which were dried without the addition of acid. In the light of this finding all but one of the subsequent assays of cow rumen contents for riboflavin, as well as the other vitamins under study were made on material which had been treated with both alcohol and acid as soon as it was removed from the rumen.

Riboflavin assays of sheep rumen and reticulum contents. For the riboflavin assays a modification of the method of Jukes ('37) was used. In this method the growth rate of chicks receiving basal diet 99 (Jukes, unpublished) deficient in riboflavin, supplemented with the test material, is compared with the growth rate of groups of chicks receiving the basal diet supplemented with various levels of synthetic riboflavin.[5]

Diet 99 had the following composition:

Ground barley	30 grams
Glucose [6]	37
Washed sardine meal [7]	23
Liver filtrate [8]	6 cc.
Ground limestone	1 gram
Iodized salt	0.45
$MnSO_4$	0.05
Soybean oil	3
Fish oil blend, 3000 U.S.P. units of vitamin A and 400 A.O.A.C. units of vitamin D per gram	0.3
Thiamine chloride hydrochloride	100 µg. per 100 gm. of diet

Liver filtrate used in diet 99 was freed of riboflavin as follows: 1 kg. of liver extract powder (Lilly 343) was dissolved in 5 to 6 liters of warm water and 1 liter of 95% ethanol was added to inhibit bacterial action. One kilogram of English fuller's earth was added and the mixture was stirred for 30 minutes. The fuller's earth was removed by filtration and washed with 500 cc. of warm water. The fuller's earth was discarded and the combined filtrate and washings were again treated with 1 kg. of fresh fuller's earth. The process was repeated once more and the combined filtrate and washings were concentrated under reduced pressure to a volume of 1400 cc. Unpublished experiments conducted in the Poultry Division of the University of California, Davis, have shown that such preparations when fed at a level of 6% in diet 99,

[5] Merck and Company.

[6] See footnote 4, page 529.

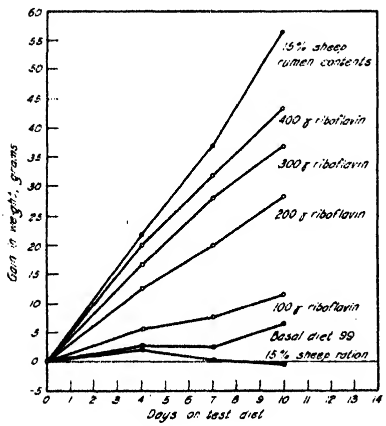
[7] See footnote 3, page 529.

[8] Ely Lilly and Company, Indianapolis, kindly supplied the liver extract powder (Lilly 343) from which the filtrate was prepared.

supplemented with riboflavin, supply sufficient pantothenic acid and possibly other as yet unidentified water-soluble factors for normal growth of the chick.

RESULTS

In the first assay the dried sheep rumen and reticulum contents were fed at a level of 15% and in the second at a level of 5%. The results obtained are summarized in figures 1 and 2. Figure 1 shows that in the first trial the sheep rumen and reticulum contents were fed at a level too high to furnish a satisfactory assay, the growth made by this group exceeding



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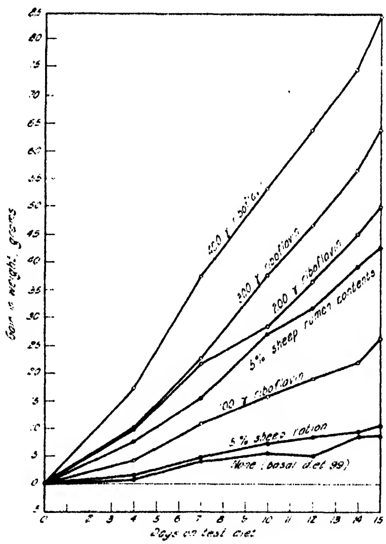

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Fig. 1 First riboflavin assay of sheep ration and of dried rumen and reticulum contents. The chicks were fed a normal diet for the first 7 days, then depleted on basal diet 99 for 8 days following which they were divided into experimental groups of ten chicks each. Supplements added to basal diet 99 are indicated opposite the curves. The amounts of riboflavin shown were added per 100 gm. of diet.

Fig. 2 Second riboflavin assay of sheep ration and of dried rumen and reticulum contents. The number of chicks used in each group and their previous treatment was the same as for figure 1 except that the birds were depleted for 7 rather than 8 days.

that made by any of the positive control groups. No riboflavin was detected in the sheep ration. Since Jukes (unpublished data) has estimated that diet 99 contains no more than 0.3 μg. of riboflavin per gram these results indicate that the experimental sheep and cow ration contained less than 0.3 μg. of riboflavin per gram.

Figure 2 shows that the growth curve for the group fed 5% sheep rumen and reticulum contents was equivalent to that which might be expected with birds receiving 165 μg. of riboflavin per 100 gm. of diet. The riboflavin content of the dried sheep rumen and reticulum contents was therefore estimated as 33 μg. per gram. The curves for the basal and 5% sheep ration groups again show that no riboflavin was detected in the sheep ration.

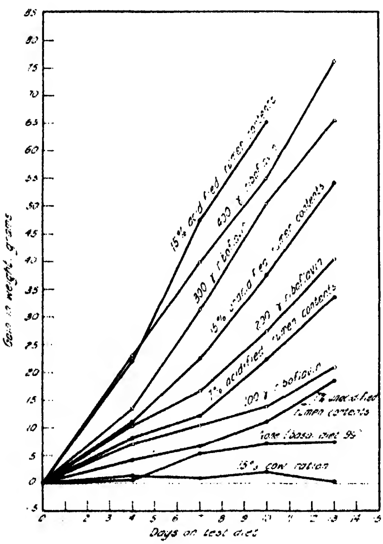
Riboflavin assays of cow ration, rumen contents and dried skim milk from fistulated cow no. 557. Two riboflavin assays of dried rumen contents and one of dried skim milk from cow no. 557 were completed. The method of assay was the same as described above for sheep rumen and reticulum contents. One change was made in diet 99, namely, the replacement of the liver filtrate with 7% rice bran filtrate.[9]

As previously mentioned, the first two samples of rumen contents taken from cow no. 557 were dried immediately after the addition of enough 95% ethanol to make the concentration of alcohol approximately 30%, while the succeeding samples were treated with alcohol and then with enough concentrated HCl to lower the pH to 4.5–5.0 before being dried. The reason for the addition of acid before drying is apparent from figure 3 which summarizes the results of the first riboflavin assay of cow rumen contents. In the 13-day assay period the group receiving 7% acidified rumen contents gained 26.2 gm. more per bird than the basal group as compared with a net gain of 11.2 gm. for the birds receiving 7% unacidified supplement.

[9] The rice bran filtrate used as a source of pantothenic acid was the "Chicken Riboflavin Assay Supplement" sold by the Vitab Corporation, Emeryville, California. Riboflavin is removed from the product by repeated treatments with fuller's earth.

At the higher level of 15% the difference between the effectiveness of the acidified and unacidified supplement was also marked.

These results indicate that drying the rumen contents at the natural pH (6.8–7.8) caused a distinct loss of riboflavin potency. No comparative assays were run to determine if a similar loss occurred with any of the other vitamins under study. As previously mentioned the sheep rumen and reticulum contents, found by biological assay to contain 33 μg. of



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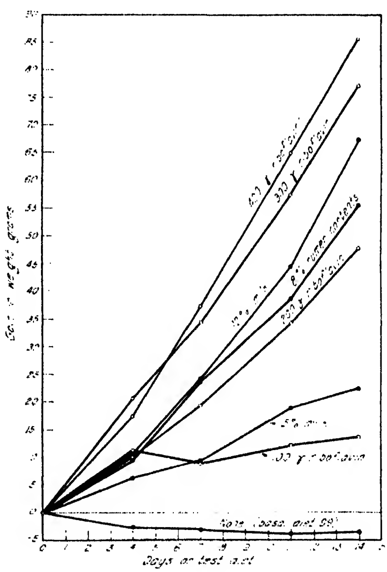

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Fig. 3 First riboflavin assay of cow ration and of dried cow rumen contents. The chicks were fed a normal diet for 8 days after hatching and were then depleted on basal diet 99 for 7 days. They were then divided into test groups of ten chicks each. Supplements added to basal diet 99 are indicated above the curves. The amounts of riboflavin shown were added per 100 gm. of diet.

Fig. 4 Riboflavin assay of dried rumen contents and of dried skim milk from cow no. 557. The chicks were fed a normal diet for 7 days and were then depleted on diet 99 for 8 days. They were then divided into experimental groups of ten chicks each. Supplements added to basal diet 99 are indicated above the curves. The amounts of riboflavin shown were added per 100 gm. of diet.

riboflavin per gram, were dried at room temperature without the addition of acid. The loss in riboflavin potency during drying may therefore have been appreciable but in view of the lower temperature used (not exceeding 30°C.) this loss would presumably be less than that found for cow rumen contents dried at 40°–50°C.

From the growth curves shown in figure 3 it was estimated that the inclusion of 7% acidified cow rumen contents in diet 99 provided at least 150 µg. of riboflavin per 100 gm. of diet. The riboflavin potency of dried cow rumen contents would therefore be at least 21 µg. per gram.

The growth curve for the group receiving 15% cow ration shows that no riboflavin was detected in the ration. Similar results are shown in figures 1 and 2.

In the second riboflavin assay of no. 557 cow rumen contents a composite mixture of equal weights of three samples taken 57, 66 and 91 days after the cow had been placed on the deficient ration was used in making up the test diets. These samples had all been acidified before drying and the mixture was fed at a level of 8%. As shown in figure 4 the group receiving this supplement made greater growth than the group supplemented with 200 µg. of synthetic riboflavin per 100 gm. of diet. From these results the average riboflavin content of the three rumen contents samples was estimated to be at least 25 µg. per gram.

In this same assay dried skim milk from cow no. 557 was tested at levels of 5% and 12%. The skim milk was prepared by concentrating under reduced pressure and evaporating to dryness in a current of air at 40°C. Equal weights of two dried skim milk preparations were used in making up the supplement to diet 99. The milk used for these preparations was collected between the seventeenth and twenty-fourth and between the forty-fifth and forty-ninth days of lactation. The growth response (fig. 4) in the groups receiving supplements of dried skim milk indicated that the riboflavin content of the skim milk was at least 20 µg. per gram on a dry basis.

In preliminary experiments the fluorescence test of Whitnah et al. ('37) was used to compare whole milk from cow no. 557 with whole milk from cows on a normal winter ration in the University herd. As reported elsewhere (McElroy and Goss, '40) the milk from the experimental cow was found to contain 1.4 ($\pm$0.15) µg. of riboflavin per gram as compared with an average of 1.5 ($\pm$0.25) µg. for the milk from twelve Jersey cows in the normal herd.

The conclusion that this cow, fed a ration practically free of riboflavin, produced milk of normal riboflavin content is further substantiated by the fact that the riboflavin values obtained by biological and fluorescence measurements are

TABLE 1

Vitamin K assay of cow ration and of dried cow rumen contents

SUPPLEMENT TO K-FREE DIET	LEVEL	NO. CHICKS	AVERAGE PROTHROMBIN TIME, SECONDS
	%		
Cow basal diet	10	6	166
Cow rumen contents	5	7	28.5
Cow rumen contents	15	7	23.8
Reference standard alfalfa	0.8	6	28.1
Practical chick mash	100	3	28.3

within the range reported for milk from cows fed a normal ration by Whitnah et al. ('37, '38), Jukes and Richardson ('38), Hodson and Norris ('39) and Hand ('39).

Vitamin K assay of no. 557 cow rumen contents and cow ration. This assay was undertaken at the suggestion of Dr. H. J. Almquist, Poultry Division, University of California, Berkeley, and we wish to acknowledge our appreciation to Doctor Almquist for having conducted the assay here reported.

The experimental ration and a composite mixture of rumen contents collected from cow no. 557, 57, 66 and 91 days after she had been placed on the diet were assayed for vitamin K by the method of Almquist and Klose ('39). The results are summarized in table 1.

These data show that the dried rumen contents of the cow was a good source of vitamin K, being about one-sixth as potent as the standard dried alfalfa. That the cow ration was practically free of the vitamin is indicated by the fact that the prothrombin time for the group supplemented with 10% of the cow ration was typical of the usual results obtained on the vitamin K-free diet alone.

Phillips, Rupel, Oleson and Bohstedt ('38) have reported improved blood-clotting times for calves when a diet apparently deficient in vitamin K was supplemented with cottonseed oil [10] and have suggested that this improvement was due to vitamin K supplied by the oil. The results of the prothrombin time assay reported in this paper indicate that under the conditions of our investigation a relatively large amount of vitamin K was synthesized in the rumen, and it would seem that the beneficial effect of cottonseed oil on blood-clotting time reported by Phillips et al. may have been due to some factor other than vitamin K.

SUMMARY

The rumen contents of four sheep fed a ration containing less than 0.3 µg. of riboflavin per gram were found to contain 33 µg. of riboflavin per gram.

Evidence for the formation of riboflavin in the digestive tract of a cow fed the same deficient ration has been presented in two ways.

1. The dried rumen contents of this animal contained approximately 25 µg. of riboflavin per gram.

2. The skim milk contained at least 20 µg. of riboflavin per gram on a dry basis, or 1.8 µg. per cubic centimeter on a fluid basis using a figure of 9% for solids in skim milk. The average daily milk production was between 9 and 10 liters and the secretion of riboflavin in the milk was therefore estimated to be between 16 and 18 mg. per day as compared with a maximum intake of 1.8 mg. per day in the feed.

[10] Wesson.

That vitamin K is not a dietary essential for the cow is indicated by the finding that, although the experimental ration was practically free from this vitamin, the rumen contents of the experimental animal proved to be a good source of the factor.

ACKNOWLEDGMENT

It is a pleasure to acknowledge the many helpful suggestions of Dr. T. H. Jukes. We are indebted to him also for his generous provision of facilities in his laboratory for conducting the riboflavin assays.

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A QUANTITATIVE STUDY OF VITAMINS IN THE RUMEN CONTENTS OF SHEEP AND COWS FED VITAMIN-LOW DIETS

II. VITAMIN B_6 (PYRIDOXINE)

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TWO FIGURES

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We have briefly reported (McElroy and Goss, '39) that the dried mixed rumen and reticulum contents of sheep, fed a ration deficient in the vitamin B complex was a good source of vitamin B_6. The investigation has now been extended to include assays for vitamin B_6 in dried rumen contents and skim milk from a cow fed the deficient ration. The complete results of the study of vitamin B_6 synthesis in these two species are reported in the present paper.

EXPERIMENTAL

The animals used, their care and ration, and the method of collection and treatment of the rumen contents have already been described (McElroy and Goss, '40 a).

Method of assay for vitamin B_6. The method used in assays for vitamin B_6 was a modification of that described by Dimick and Schreffler ('39). The diet of the positive control rats was supplemented with synthetic vitamin B_6 hydrochloride[1] instead of the crystalline factor I of Lepkovsky ('38). For the assay of sheep rumen and reticulum contents a basal rat diet

[1] Kindly furnished by Merck and Company, Rahway, New Jersey, through the kindness of Dr. D. F. Robertson.

with the following composition was used: glucose [2] 60 parts, washed casein (McElroy and Goss, '40 a) 27, hydrogenated cottonseed oil [3] 8, salt mixture (Jukes, '39), 5, and fish oil blend containing 3000 U.S.P. units of vitamin A and 400 A.O.A.C. units of vitamin D per gram, 0.2 parts.

In the assay of cow rumen contents and milk from the experimental cow this basal rat diet was changed to include 4 parts hydrogenated cottonseed oil and 4 parts lard. This change was made to avoid the possibility of a deficiency of unsaturated fatty acids in the basal diet. Since no difference was noted in the response of the rats in the two assays it is believed that the first basal ration, used for the assay of vitamin B_6 in sheep rumen material, was satisfactory even though no precaution was taken to provide additional unsaturated fat.

The test animals used were female rats of the Long-Evans strain, weaned at 21 days and placed in groups of three to four in cages with raised wire bottoms. For the first 2 weeks after weaning they were fed only the basal diet. From the third week on each individual was fed 0.2 cc. of a supplement containing 15 µg. of thiamine chloride hydrochloride and 30 µg. of riboflavin dissolved in a 10% solution of ethanol. Factor II was supplied from the beginning of the fifth week to the end of the assay. In the assay of sheep material a liver filtrate [4] was used for this purpose which had been prepared by five treatments with fuller's earth after the method of Lepkovsky, Jukes and Krause ('36). After concentration under reduced pressure the filtrate was treated with 8 volumes of 95% ethanol. The precipitate brought down by this treatment was discarded and the final filtrate was again concentrated so that 1 cc. of filtrate represented 2 gm. of the original liver extract powder. No tests were run to determine the potency of this preparation but it is believed that the dose fed (0.5 cc. daily) provided ample factor II for normal growth of the rat.

[2] Cerelose.

[3] Crisco.

[4] The liver extract powder (Lilly 343) from which the filtrate was prepared was kindly furnished by Ely Lilly and Company, Indianapolis.

In the assay of cow material the liver filtrate used was prepared from liver extract 70 A.S.[5] by similar treatment with fuller's earth and alcohol. This preparation was fed at a level of 0.2 cc. daily since preliminary experiments showed that this amount supplied sufficient factor II to satisfy the growth requirement of the rat.

The rats were maintained on the depletion diet until growth had practically ceased and dermatitis had developed. In addition to the usual dermatitis of the face, ears and paws a "ring-tailed" condition (McElroy and Goss, '40 b) developed in a number of the test animals.

At the end of the depletion period of about 7 weeks the rats were divided into uniform test groups. All groups continued to receive the previous supplements of thiamine, riboflavin and liver filtrate preparation, while the two positive control groups received a further daily supplement of 0.2 cc. of an aqueous solution containing 3 and 10 μg. respectively of synthetic vitamin B_6 hydrochloride.

Results of assay of sheep ration and of sheep rumen and reticulum contents. The growth made by the different groups in this assay is shown in figure 1. A comparison of the growth curves for groups V and VI indicates that the sheep ration probably contained a small amount of vitamin B_6. There was some evidence of healing of the dermatitis in three members of the group fed the basal diet supplemented with 20% sheep ration; of the other four, three remained unchanged and one developed a more severe dermatitis.

The growth curves for groups I and III show that the rumen and reticulum contents contained much more vitamin B_6 than did the sheep ration. Group I, whose diet was supplemented with 20% dried sheep rumen and reticulum contents, gained an average of 60 gm. per rat in 13 days, and exhibited rapid and uniform healing of the dermatitis throughout the lot. Feed consumption in this group was very high, and since only a limited amount of the rumen material was available these

[5] Kindly furnished by the Armour Laboratories, Chicago, Illinois, through the courtesy of Dr. E. F. Pike.

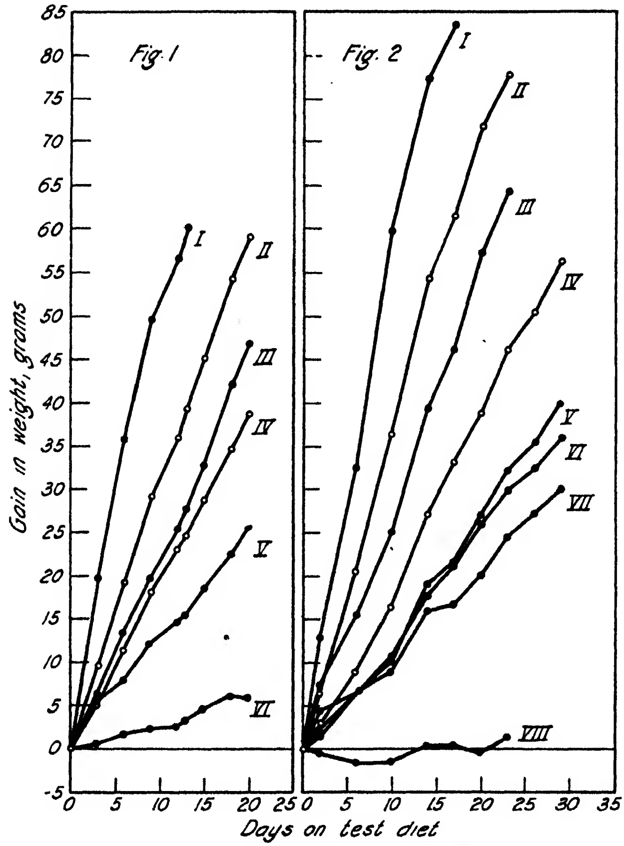

Fig. 1 Growth curves for rats in vitamin B_6 assay of sheep ration and of dried sheep rumen and reticulum contents. Seven female rats were included in each group. The basal diets of the groups were supplemented as follows: I. 20% dried sheep rumen and reticulum contents. II. 10 μg. vitamin B_6 daily. III. 5% dried sheep rumen and reticulum contents. IV. 3 μg. vitamin B_6 daily. V. 20% sheep ration. VI. None (basal only).

Fig. 2 Vitamin B_6 assay of cow ration and of dried rumen contents and skim milk from cow no. 557. Six female rats were included in groups I, III, IV and VIII, and five in groups II, V, VI and VII. The basal diets of the groups were supplemented as follows: I. 15% dried rumen contents. II. 10 μg. vitamin B_6 daily. III. 5% dried rumen contents. IV. 3 μg. vitamin B_6 daily. V. 4 cc. concentrated skim milk daily. VI. 15% cow ration. VII. 2 cc. concentrated skim milk daily. VIII. None (basal only).

rats were killed on the thirteenth day of the assay. Group III, fed 95 gm. of the basal diet plus 5 gm. of dried rumen and reticulum contents gained an average of 46.7 gm. per rat over the 20-day assay period as compared with 38.7 and 58.9 gm. for the groups receiving a daily supplement of 3 and 10 µg. of synthetic vitamin B_6 hydrochloride. The degree of healing of the dermatitis in the group fed 5% rumen and reticulum contents was intermediate between that observed in the two positive control groups.

Group III, fed 5% dried sheep rumen and reticulum contents, ate an average of 9.8 gm. of feed, or the equivalent of approximately 0.5 gm. of test substance per rat daily. Since the consumption of this amount of rumen and reticulum material caused growth and healing which might be expected with rats receiving 5 µg. of vitamin B_6 daily, it was estimated that the dried rumen and reticulum contents contained approximately 10 µg. of vitamin B_6 per gram.

Results of assay of cow ration and of dried rumen contents and skim milk from fistulated cow no. 557. The dried rumen contents of fistulated cow no. 557 was tested at 5% and 15% levels. Aliquot portions of rumen material removed 57, 66 and 91 days after the cow had been placed on the deficient ration were used in making up the test diets. In testing the milk a composite sample collected during the forty-fifth to the forty-ninth days of lactation was used. This sample had previously been skimmed in an ordinary cream separator and then concentrated under reduced pressure to one-third of its initial volume. The test groups received 2 cc. and 4 cc. per rat daily of this preparation or the equivalent of 6 cc. and 12 cc. of skim milk.

The results of the assay are summarized in figure 2. The growth response in group IV supplemented with 3 µg. of vitamin B_6 per day was practically identical with that of the corresponding group IV in figure 1. The degree of healing of dermatitis obtained with this amount of the pure vitamin was also duplicated. When 10 µg. of vitamin B_6 was fed, healing

progressed at the same rate in both assays but growth was somewhat more rapid in the second one.

Group VI (fig. 2), whose diet was supplemented with 15% cow ration, made almost the same growth as the group fed 20% of the same ration in the assay conducted on sheep material (fig. 1). In both assays the sheep or cow ration groups failed to make as much growth as the lowest positive control group (3 μg. vitamin B_6 per day) and only a rough estimate may be made for the vitamin B_6 content of the ration fed to the experimental sheep and cows. By assuming that the growth made was equivalent to that which would be made by rats fed 2 μg. of vitamin B_6 per day, and calculating from feed consumption records the amount of sheep (or cow) ration consumed, it was estimated that the experimental sheep and cow ration contained between 1 and 1.5 μg. of vitamin B_6 per gram.

From figure 2, groups II, III and IV, it was estimated that the rats in group III obtained at least 5 μg. of vitamin B_6 per day. The average feed consumption in this group was 12.0 gm. or 0.6 gm. of rumen contents per rat daily. The vitamin B_6 content of the dried cow rumen material was thus estimated to be at least 8 μg. per gram, which is about the same potency as estimated for dried sheep rumen and reticulum contents.

As shown in figure 2, the group fed 15% cow rumen contents gained weight more rapidly than the group whose diet was supplemented with 10 μg. of vitamin B_6. The appetite of these rats improved immediately when they were placed on the test diet, as is indicated by the fact that they gained an average of 13 gm. per rat during the first 2 days of the assay. Their average daily feed consumption for the assay period was 16 gm. per rat. The rats of this group were indistinguishable from normal animals when they were removed from the experiment on the seventeenth day. It was observed that, in all groups supplemented with either sheep or cow rumen material, food wastage became negligible as soon as the supplement was added.

The growth curve and healing response of group V, which received 4 cc. of concentrated skim milk per rat daily, indicated that these animals were receiving about 2 μg. of vitamin B_6 per day. Since the milk was concentrated 3:1, the vitamin B_6 content of the original skim milk was estimated as 0.17 μg. per cubic centimeter. No recent vitamin B_6 assays of cow's milk have come to our attention, but György ('36) has reported vitamin B_6 assays of cow's milk in which a daily dose of 15 cc. of milk resulted in an average weekly increase of 14 gm. per rat. The corresponding figure from the present assay was 10 gm. per rat with a dose equivalent to 12 cc. of skim milk. Since György provided only vitamin B_1 and riboflavin in his test

TABLE 1

A comparison of the growth of rats depleted of vitamin B_6 for 71 days and then fed for 21 days the depletion diet plus 20% of either commercial skim milk powder or skim milk powder made from the milk of cow no. 557

SUPPLEMENT	RAT NO.	WEIGHT IN GRAMS AT 3-DAY INTERVALS								GAIN IN GRAMS	
		Initial	3	6	9	12	15	18	21	Total	Avge. daily
Commercial	1	91	97	103	110	112	117	121	123	32	1.5
skim milk	2	87	88	93	99	104	110	119	127	40	1.9
powder	3	54	52	Dead 5th day							
Cow no. 557	4	68	71	79	92	97	100	109	117	49	2.3
skim milk	5	90	105	107	110	118	123	128	140	50	2.4
powder	6	124	125	135	140	148	151	158	168	44	2.1

diet, his results are not strictly comparable with those of the present investigation, in which the test diet was supplemented with "factor II" (Lepkovsky, Jukes and Krause, '36) in addition to thiamine and riboflavin. However, a comparison of the results obtained at least suggests that the experimental cow, fed a ration deficient in vitamin B_6, produced milk containing a normal amount of this vitamin.

A direct comparison was made of the vitamin B_6 content of commercial skim milk powder prepared by the drum process and that of skim milk powder prepared from milk collected from our experimental cow between the seventeenth to twenty-sixth and the forty-fifth to forty-ninth days of lacta-

tion. This skim milk powder was prepared by first concentrating under reduced pressure and then drying to a constant weight before a current of air at approximately 40°C.

The six rats (group VIII, fig. 2) included in the basal group of the vitamin B_6 assay just described were used for this assay. These animals had been on the depletion diet for 71 days and were consequently in very poor condition. Two of them, nos. 3 and 4, were not expected to recover when their diet was changed to include 20% skim milk powder. As shown in table 1, rat no. 3 died 5 days after the diet was changed but no. 4 recovered and made as rapid growth as any of the other animals. Although the number of rats included is small, table 1 shows that the skim milk powder prepared from the milk of cow no. 557, fed a ration deficient in vitamin B_6, contained fully as much of this vitamin as did the commercial product.

DISCUSSION

The ratio of vitamin B_6 potency of rumen contents to the potency of the ration was found to be between 6 and 8 to 1. The question arises whether this represents merely a concentration of the vitamin due to differential rates of passage of different nutrients from the rumen, or whether there has been a true synthesis related to the growth of the rumen flora. In our opinion there are reasons why the latter view should be favored. The amount of vitamin B_6 present in the milk of cow no. 557 was apparently normal which indicates that the cow received enough vitamin to meet her maintenance metabolic needs and was able in addition to transfer the normal amount through the mammary gland into the milk. The chief weakness of this argument resides, of course, in our lack of knowledge of the actual requirement of the cow for vitamin B_6.

Such data as were obtained concerning the differential rates of passage of materials from the rumen are open to different interpretations. For example, the ration contained 0.42% fat

and the rumen contents of the fistulated cow 2.56%. The corresponding figures for protein were 15.2% and 29.8%. On the other hand, the nitrogen-free extract values were 59.0% and 17.8% respectively. In view of its solubility it might be argued that vitamin B_6 would be more comparable to the nitrogen-free extract material than to the protein or fat in its rate of passage from the rumen, and from this point of view, one would be led to conclude that the vitamin had been formed in the rumen.

SUMMARY

The dried rumen and reticulum contents of four sheep fed a ration containing between 1 and 1.5 μg. of vitamin B_6 per gram were found to contain approximately 10 μg. of vitamin B_6 per gram.

The dried rumen contents of a fistulated cow fed the same ration was estimated to contain 8 μg. of vitamin B_6 per gram.

Milk from the cow fed the deficient ration contained at least as much vitamin B_6 as milk from cows fed a normal ration.

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STUDIES ON THE METABOLISM OF NICOTINIC ACID IN THE SHEEP [1]

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TWO FIGURES

While the significance of nicotinic acid in the nutrition of man, dogs and pigs is well established, information on its dietary importance for ruminants is limited. On the basis of growth studies (Pearson, Schmidt and Mackey, '39) evidence was presented indicating that nicotinic acid is not a dietary essential for sheep. McElroy and Goss ('40) reported an increase in the nicotinic acid of the rumen contents of a cow fed a diet deficient in this constituent. Winegar, Pearson and Schmidt ('40) reported that sheep restricted to a diet low in nicotinic acid continued to excrete substantial amounts in the urine. Thus it appears that nicotinic acid is formed in the body of sheep, since animals for which it is a dietary essential virtually cease to excrete it when restricted to a deficient diet. We have continued our investigations on the metabolism of nicotinic acid in ruminants and have studied the effect of the intake on the amounts in the blood and urine, the rate of elimination and recovery of massive doses.

EXPERIMENTAL

Seven lambs approximately 10 weeks of age and weighing between 9 and 12 kg. were placed on a diet having a percentage

[1] Published with the approval of the Director of the Texas Agricultural Experiment Station as technical contribution no. 615.

composition of regenerated cellulose 20, brewers' rice 50.5, purified casein [2] 9, yellow corn 16, cow peas 2.5 and salts mixture (Hughes, '38) 2. Vitamins A and D in addition to that furnished through natural sources were supplied by oleum percomorphum or other concentrated fish oils at weekly intervals. Originally riboflavin was included in the diet but was later omitted when it was reported (McElroy and Goss, '39) that this vitamin is synthesized in the rumen of sheep.

The nicotinic acid content of the various components of the diet was determined photometrically by the technique of Bandier ('39 a) with the modification that aniline was used in place of metol. On the basis of the chemical assay the diet contained 0.6 mg. of nicotinic acid per 100 gm.

The diet was also assayed with dogs for its blacktongue producing properties. In order to insure an adequate caloric intake for the dogs the regenerated cellulose was omitted. The cellulose was included in the diet for the sheep merely to provide the necessary bulk. This alteration in the diet has no material bearing on the nutritional value as lignified cellulose is almost entirely undigestible even by ruminants (Crampton and Maynard, '38). Dogs fed this diet showed a marked decrease in their urinary nicotinic acid and developed typical blacktongue symptoms.

Growth response. Early in the experiment two lambs died from undeterminable causes leaving only five on which complete data are available. The growth response of all of the lambs was very unsatisfactory as there was only a slight increase in weight during an experimental period of 214 days. This was probably due to the unpalatability of the diet and the resulting low feed consumption which averaged only 408 gm. daily as compared with 934 gm. for lambs receiving a similar diet except that corn was used in place of brewers' rice (Pearson et al., '39). During the last 160 days lamb no. 447 was fed 10 gm. of dried brewers' yeast per day. Since the yeast failed to bring about any response in either appetite or growth further support is afforded the belief that the poor growth

[2] Glaxo.

performance was not due to a deficiency of members of the vitamin B complex. The unfavorable growth response does not affect the validity and interpretation of the chemical data as will be shown later by comparison with results obtained with lambs on a standard stock diet.

Blood studies. At monthly intervals the nicotinic acid content of the blood was determined photometrically by the procedure of Pearson ('39). This method with the slight modification that the nicotinic acid is adsorbed on fuller's earth has been used successfully with blood by Villela ('40).

TABLE 1

Nicotinic acid content of blood

LAMB NUMBER	DIET	RANGE	AVERAGE
		mg./100 ml.	*mg./100 ml.*
418	Deficient	0.48–1.52	0.93
420	Deficient	0.63–1.18	0.87
443	Deficient	0.65–1.70	1.00
488	Deficient	0.59–1.14	0.84
447	Deficient	0.72–1.27	1.01
447	Deficient + yeast	0.60–1.27	0.92
523	Stock [1]		0.61
537	Stock [1]		1.54
550	Stock [1]		0.81
558	Stock [1]		0.62
3445	Stock [1]		0.97

[1] One analysis.

While there was considerable variation in the values for individual lambs during the course of the experiment, the magnitude was about the same as that previously reported (Pearson, '39) for normal lambs on a similar diet. There was no consistent trend in the nicotinic acid content of the blood as the experiment progressed. For this reason only the range and average values for the 7-month period are presented in table 1. There was no consistent increase in the nicotinic acid level of the blood of lamb no. 447 after yeast was included in the diet. Furthermore, there is no essential difference between the values of the lambs on the deficient diet and those

receiving a stock diet consisting of corn, alfalfa hay, cottonseed meal, and a limited amount of green grass. These observations are of interest in connection with those of Ballif et al. ('39) who found no reduction in the nicotinic acid amide content of the blood of ten pellagrins. Likewise there appears to be no decrease in the coenzyme content of the blood of pellagrins (Kohn, '38), dogs and pigs fed deficient diets (Kohn, Klein and Dann, '39; Axelrod, Madden and Elvehjem, '39). On the other hand it has been shown that the coenzyme content of the blood can be increased by feeding nicotinic acid (Kohn, '38; Axelrod, Gordon and Elvehjem, '40). The latter workers fed as much as 1.8 gm. in a period of 16 hours. The ingestion of this relatively large amount as compared with the intake of the lambs on the stock diet may be a factor in accounting, at least in part, for the increase in the coenzyme content of the blood of pellagrins and the failure to observe a difference in the nicotinic acid level of the blood of lambs on the deficient diet and those receiving a stock diet or a supplement of yeast. In fact it will be shown later that the ingestion of large doses of nicotinic acid increases by severalfold the level in the blood. A second factor, as will be pointed out later, is that nicotinic acid is synthesized in the body of this species; thus adequate amounts are available to maintain normal levels in the blood.

Urinary nicotinic acid. The determination of the nicotinic acid of the urine of sheep on a deficient diet (Winegar, Pearson and Schmidt, '40) was made on the unhydrolyzed urine by the aniline and cyanogen bromide reaction as described by Pearson and Winegar ('40). Since this work was done there have appeared several papers (Bandier, '39 b; Harris and Raymond, '39; Swaminathan, '39) showing that a part of the nicotinic acid may be excreted as nicotinamide, nicotinuric acid and the codehydrogenases. These conjugated forms can be converted to the free nicotinic acid by alkaline hydrolysis. It was, therefore, of interest to study the effect of alkaline hydrolysis on the nicotinic acid values of the urine of normal

sheep with a view to the possible reinterpretation of the data obtained from the sheep on the deficient diet.

The urine was hydrolyzed and the nicotinic acid determined by the method of Harris and Raymond ('39) with the modification that aniline was used in place of para-aminoacetophenone. The urine was collected quantitatively over a 24-hour period from lambs weighing between 17 and 23 kg. The figures in table 2 are average values for 3 consecutive days. Subjecting the urine to alkaline hydrolysis for 30 minutes resulted in increases of from 12.6 to 31.6% in the nicotinic acid values.

TABLE 2

Nicotinic acid values in urine before and after hydrolysis

LAMB NUMBER	UNHYDROLYZED	HYDROLYZED	INCREASE FROM HYDROLYSIS
	mg./24 hr.	*mg./24 hr.*	*%*
523	3.27	4.10	25.4
527	2.67	3.45	29.2
528	2.41	2.99	24.1
532	2.06	2.32	12.6
537	3.32	4.04	21.7
545	2.17	2.66	22.6
550	1.93	2.54	31.6
558	5.32	6.22	16.9
3439	5.74	6.76	17.8
Average	3.21	3.90	21.4

The average increase observed in the urine collected from nine lambs was 21.4%. From these data it is apparent that a part of the nicotinic acid is excreted as conjugates. The increase in nicotinic acid values due to hydrolysis is, however, much less for sheep urine than that reported for human urine. Bandier ('39 b) found that alkaline hydrolysis of human urine gave values from 164 to 333% higher than unhydrolyzed urine. Swaminathan ('39) reported increases of from 45 to 106% after alkaline hydrolysis.

These investigators did not indicate whether or not their subjects were smokers. This may be a factor in accounting for difference in the relative amounts of free and conjugated forms of nicotinic acid excreted in man and sheep. Ackermann ('12)

found nicotinuric acid in the urine of a dog that had received nicotinic acid orally, while Harris and Raymond ('39) observed that a heavy smoker excreted approximately twice as much nicotinic acid per day as did non-smokers.

The relatively small increase in the nicotinic acid values after the hydrolysis of sheep urine affords no basis warranting the revision of the previous conclusions of the authors that nicotinic acid is synthesized in the alimentary tract or body of sheep. This conclusion was based on the fact that lambs restricted to a deficient diet continue to excrete in their urine essentially as much nicotinic acid as lambs on a standard well balanced growing diet. On the other hand dogs used to assay the deficient diet excreted in unhydrolyzed

TABLE 3

Recovery of nicotinic acid after administration of test dose

LAMB NUMBER	AMOUNT INGESTED	COLLECTION PERIOD	TOTAL EXCRETION	INITIAL EXCRETION	NET RECOVERY	PER CENT RECOVERED
	gm.	*hr.*	*mg.*	*mg.*	*mg.*	
523	8.20	73	7576	16.0	7560	92.2
532	6.20	36	3673	8.0	3665	59.1
537	10.45	50	4725	17.0	4708	45.1
550	8.38	50	3594	10.6	3583	42.7
558	9.94	50	7107	26.0	7081	71.2

samples of urine approximately fifteen times as much nicotinic acid daily on a stock diet as they did after being fed the experimental diet long enough to develop typical blacktongue symptoms.

Excretion of nicotinic acid following massive doses. The recovery of nicotinic acid in the urine after the ingestion of a single large dose was studied with five normal lambs that received a stock diet. The urine was collected for 3 consecutive days prior to the administration of the nicotinic acid. The nicotinic acid was given by mouth in gelatin capsules. The urine was collected for various periods after the ingestion of the test dose and the nicotinic acid determined on the hydrolyzed samples.

The data on the recovery of ingested nicotinic acid are summarized in table 3. The recovery of nicotinic acid in the urine

ranged from 42.7 to 92.2%. The recovery of nicotinic acid in the urine of sheep is considerably higher than has been reported for man when smaller amounts were ingested. Bandier ('39 b) fed 90 mg. and recovered 13.9% within 6 hours at which time the excretion had returned to normal. Swaminathan ('39) fed ten normal individuals each 100 mg. of nicotinic acid and recovered between 14.0 and 37.9% during the first 24 hours. Of 1 gm. of nicotinic acid administered subcutaneously to a rabbit Ritsert ('39) recovered from the urine 39.0% over a period of 120 hours. When the same amount was given orally 31.0% was recovered.

TABLE 4

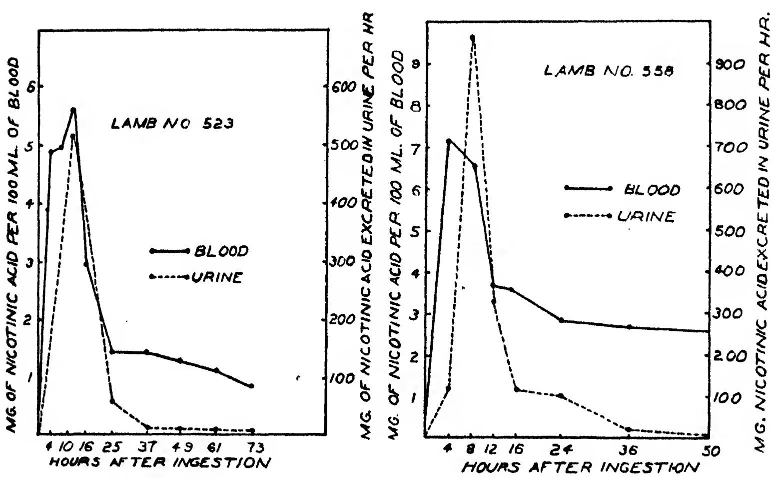
Rate of excretion of nicotinic acid after test dose

LAMB NUMBER	AMOUNT INGESTED	HOURS AFTER INGESTION	COLLECTION PERIOD	TOTAL EXCRETION DURING PERIOD	EXCRETION PER HOUR
	gm.		*hr.*	*mg.*	*mg.*
537	10.45	4	4	96	24.0
		8	4	1175	293.7
		12	4	887	221.7
		24	12	2137	178.1
		36	12	280	23.3
		50	14	150	10.7
550	8.38	8	8	1662	207.7
		16	8	977	122.1
		24	8	649	81.1
		36	12	166	13.8
		50	14	140	10.0

Rate of excretion. The urine of four of the lambs was collected at frequent intervals in order to obtain information on the rate of elimination of large doses of nicotinic acid. Originally it was planned to collect the urine at 4-hour intervals for the first 16 hours, followed by 8-, 12- and 14-hour periods. However, since micturition did not always occur during the 4-hour periods it was necessary to vary this plan. While micturition is more or less irregular especially when animals are confined to metabolism cages there is no reason to believe that renal secretion does not proceed at a relatively constant rate as under normal conditions. Thus in table 4 the rate

of excretion of nicotinic acid has been calculated on an hourly basis for the various collection periods. In order to conserve space the detailed data are shown in table 4 for only two of the lambs while the data for the other two are presented graphically in figures 1 and 2.

Of the total amount of nicotinic acid excreted during the 50 hours the urine was collected, lamb no. 550 excreted 46.2% during the first 8 hours. The comparable figures for animals



Figs. 1 and 2 Nicotinic acid content of the blood and urinary excretion of lamb nos. 523 and 558 following the ingestion of 8.20 and 9.94 gm. of nicotinic acid respectively.

558 and 537 are 60.8 and 26.9% respectively. The lower amount excreted by no. 537 is reflected in the higher concentration in the liver as will be brought out later. Lamb no. 523 did not micturate during the first 8 hours. The first collection of urine from this animal was made on the twelfth hour. During this period the excretion of nicotinic acid was 88.5% of the total for the first 49 hours. From this it appears that in sheep large doses of nicotinic acid are rapidly eliminated from the body by the kidney. This agrees with observations

of Ritsert ('39) who administered 1 gm. of nicotinic acid subcutaneously to a rabbit and during the first 24 hours recovered 85.1% of the total excreted over a 120-hour period. When the same amount was given by mouth 65.3% of the total excreted was recovered in the urine during the first 24 hours.

Free and conjugated nicotinic acid in urine following ingestion of massive doses. By determining the nicotinic acid values of urine on hydrolyzed and unhydrolyzed samples following the ingestion of a massive dose, information has been obtained on whether under such conditions nicotinic acid is excreted as the free acid or as conjugates. The effect of hydrolysis is shown in table 5. The total time during which

TABLE 5

Effect of alkaline hydrolysis on nicotinic acid values of urine after ingestion of massive dose

LAMB NUMBER	TOTAL NICOTINIC ACID EXCRETED		INCREASE FROM HYDROLYSIS
	Unhydrolyzed	Hydrolyzed	
	gm.	*gm.*	%
523	7.24	7.58	4.7
537	4.19	4.71	12.4
550	3.48	3.69	3.2
558	6.26	7.11	13.6

the urine was collected is the same as recorded for these animals in table 3. The hydrolyzed urine gave values of from 3.2 to 13.6% higher than the unhydrolyzed. Since nicotinamide gives only 20% of its maximum color if unhydrolyzed (Kodicek, '40) it appears that a part, though a relatively small percentage, of the ingested nicotinic acid was converted to some conjugated form in the body. A comparison of the data in tables 2 and 5 indicates that a much smaller percentage of a large dose of nicotinic acid is excreted as conjugates than occurs under normal conditions.

Relationship of blood and urinary nicotinic acid following massive dose. In connection with the study of the urinary excretion, blood was drawn at frequent intervals following

the test dose. The normal nicotinic acid level of the blood was determined while the animals were on the stock diet and prior to the administration of the test dose. The relationship of the blood nicotinic acid to the urinary excretion is shown graphically for two animals in figures 1 and 2. The rate of urinary excretion of nicotinic acid is calculated on an hourly basis for each collection period.

From the figures it is apparent that there is a close correlation between the urinary excretion and the concentration of nicotinic acid in the blood. The increases in the urinary levels are, of course, of a far greater magnitude than those in the blood. Similar relationships were observed with lambs nos. 537 and 550, but the detailed data have been omitted in order to conserve space. The increases in the nicotinic acid level of the blood above the normal level were 8.3, 3.9, 5.9 and 11-fold for lambs 523, 537, 550 and 558 respectively.

The rapid fall of the nicotinic acid level of the blood parallels closely the decline in the rate of urinary excretion. The blood values of lamb no. 558 did not return to normal until approximately 73 hours after the administration of the test dose. Fifty hours after the administration of the test dose to lamb no. 558 the nicotinic acid level of the blood was still 4.4 times the normal level. These observations are in agreement with the findings of Axelrod et al. ('40) that the coenzyme I content of the blood can be increased from 1.5 to 3-fold by feeding nicotinic acid.

Observations on the liver. Three lambs were killed by exsanguination 50 hours after ingestion of the test dose and the nicotinic acid of the liver determined by the method of Bandier ('39 a) with the modification that metol was replaced by aniline as in the blood and urine analysis. While the number of observations recorded in table 6 is too small to permit any conclusions they may serve to draw attention to points that might merit further study. During the 50-hour collection period lamb no. 550 excreted in the urine 42.7% of the ingested nicotinic acid. The liver contained 10.6 mg. of nicotinic acid per 100 gm. of fresh tissue. Histological examination showed

marked fatty degeneration of the liver which may account for its low nicotinic acid content. Since the livers of both of the other lambs appeared normal it is not believed that the fatty degeneration was caused by the massive dose of nicotinic acid. However, a final answer to this problem must await further studies. Forty-five per cent of the nicotinic acid ingested by lamb no. 537 was recovered in the urine, and the liver contained 44.0 mg. per 100 gm. of fresh tissue. The corresponding data for lamb no. 558 are 71.2% recovered in the urine and 23.4 mg. of nicotinic acid per 100 gm. of fresh liver. On the basis of the nicotinic acid content for normal sheep liver of 20.0 mg. per 100 gm. of fresh liver (Kodicek, '40) and values of the same order for ox and pork liver (Bandier, '39 a; Karrer and Keller, '39; Kringstad and Naess, '39) it appears that the ingestion of a single massive dose of nicotinic acid materially increases the nicotinic acid content of the liver. The extent to which the liver and other organs can be enriched in nicotinic acid by the ingestion of liberal amounts might merit investigation.

TABLE 6

Nicotinic acid content of livers

NUMBER	INGESTED	RECOVERED IN URINE	PER 100 GM. FRESH LIVER	TOTAL
	gm.	*%*	*mg.*	*mg.*
537	10.45	45.1	44.0	135.5
550	8.38	42.7	10.6	51.9
558	9.94	71.2	23.4	81.4

SUMMARY

There is no essential difference in the nicotinic acid level of the blood of lambs fed a diet low in nicotinic acid and those fed a stock diet. Lambs restricted to the deficient diet for several months continue to excrete in their urine approximately as much nicotinic acid as those on a stock diet.

Subjecting the urine of normal animals to alkaline hydrolysis increased the nicotinic acid values 21.5%. Since the in-

crease after the ingestion of a single large dose of nicotinic acid is much smaller, it appears that only a relatively small percentage is conjugated in the body under these conditions. Following the ingestion of a single large dose of nicotinic acid 42.7 to 92.2% was recovered in the urine.

Fifty hours after the ingestion of a large dose of nicotinic acid the amount in the liver was materially augmented. The extent of this increase appears to bear some relationship to the amount excreted.

Following the ingestion of a massive dose the nicotinic acid level of the blood increases severalfold. This rapid increment and the subsequent decline parallels closely the rate of urinary excretion.

Acknowledgments are made to Anheuser-Busch Inc., F. E. Booth Company Inc., and Merck & Company for materials used in the investigation and to Dr. H. A. Smith for histological examination of the livers.

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PANTOTHENIC ACID REQUIREMENT OF THE RAT

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THREE FIGURES

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It has long been recognized that rats maintained on synthetic diets supplemented with thiamin and riboflavin need, for normal growth and development, additional factors which are present in liver and yeast. Lepkovsky, Jukes and Krause ('36) separated from liver concentrates two fractions, one of which later proved to be vitamin B_6. The other fraction, factor II or filtrate factor, was found necessary for the growth of rats and was effective in curing the chick dermatitis, previously recognized as a vitamin deficiency by Elvehjem and Koehn ('35). Later, Jukes ('39 a, b) and Woolley, Waisman and Elvehjem ('39) reported that this chick antidermatitis factor was probably identical with pantothenic acid (Williams, '39). Progress in the preparation of pantothenic acid has made it possible to investigate its nutritional effect in rats.

Subbarow and Hitchings ('39) observed a marked growth response in rats, on a basal diet supplemented with the crystalline factors of the vitamin B complex, when fed a liver concentrate containing pantothenic acid. Oleson, Woolley and Elvehjem ('39) reported that the rat requires in addition to the available crystalline factors of the B complex, an alkali labile factor which is probably pantothenic acid. Recently György, Poling and Subbarow ('39) reported that a crude zinc salt of pantothenic acid was curative for skin lesions which developed on diets devoid of the filtrate factor.

In the Merck Research Laboratories, pantothenic acid was made from pure "natural" α-hydroxy-β, β-dimethyl-gamma-butyrolactone and β-alanine, and samples of it were made available by Doctor Stiller and Doctor Keresztesy for this study. The potency of the preparation was determined by its growth stimulating effect on Lactobacillus casei (Snell, Strong and Peterson, '37) in direct comparison with a pantothenic acid standard furnished by Dr. R. J. Williams. Pantothenic acid made by such a procedure is free from any other nutritional factors which might be present as impurities in natural pantothenic acid concentrates.

The animals used in these experiments were maintained on a vitamin B complex free diet consisting of dextrose, 68%; casein, 18%; hydrogenated cottonseed oil,[1] 8%; salt mixture, 4%; cod liver oil, 2% and supplemented with 40 μg. each of thiamin, riboflavin and vitamin B_6 and 5 mg. of choline chloride per rat per day. In part of the experiments nicotinic acid amide was also added in daily doses of 0.5 mg.

Twenty-one-day-old rats placed on this regimen grew at a rate of approximately 1.5 gm. per day throughout the first 3 weeks, at which time the weight became stationary at 50 to 65 gm. If no pantothenic acid was administered, the survival time on this diet was from 25 to 60 days. Starting at the second week, the rats developed a rough, thin fur, excessive nasal secretion, rusty spots on the fur, sores around the mouth and blood-caked whiskers. Change in the appearance of the fur was invariably the first symptom. The long hairs of the cover coat became loose and were readily shed. The fur lost its lustre and took on the appearance of that of a very young animal. Thinning of the soft short hair occurred to such an extent that blowing at the fur readily exposed the bare, rough and scaly skin. This process started usually at the groin and on the head just back of the ears, but soon became generalized over the body. Some animals approached complete baldness. Excessive nasal secretion occurred in the

[1] Crisco.

majority of the animals and was occasionally found to contain blood. Sores around the mouth frequently developed. The rats showed rusty spots on the fur especially on the head and on the back. During the third week the whiskers showed some bloody discoloration which later developed into blood-tinged cakes on the whiskers. It cannot be stated definitely whether the blood on the whiskers was caused by bleeding into the hair follicles or by the animals rubbing it onto the whiskers from sores around the mouth. The phenomenon of blood-caked whiskers occurred in the majority of the animals, sometimes to such an extent that the rat assumed the bearded appearance of a walrus. Deficiency symptoms on the eyelids were observed in only 10 to 20% of the animals. They began with an exudation on the lids which kept the eye stuck shut. Later these animals showed denudation around the eye, a halo resembling spectacles. In addition to the findings noted above, a large percentage of the rats showed, on autopsy, numerous petechiae under the skin and a hemorrhagic condition of the adrenals and intestines. Histopathological studies are in progress and will be reported later.

Curative effect following single doses

Single doses of 100, 200, 300, 600 and 840 μg. respectively of pantothenic acid were administered orally to groups of six to ten rats which had been on the deficient diet for 30 to 40 days and exhibited the above described symptoms. Daily records of weight and symptoms were kept in all experiments.

A single dose of 100 μg. was without noticeable effect. The response to single doses of 200, 300 and 600 μg. was irregular. About half of the animals on each of these dose levels gained 1 to 2 gm. per day over a period of 3 to 4 days; the other half failed to show any significant weight gain and died probably because single doses within this range were not sufficient to influence a well advanced deficiency. However, with a single dose of 840 μg. only three out of ten rats died and the remaining animals showed a uniform growth response of about 3 gm.

per day for 4 to 5 days. Growth then slowed down and the weight curve leveled off. A second dose of 840 µg., administered 6 days after the first, was followed by an almost identical response. Again the weight became stationary after 6 days. A third dose of 840 µg. administered on the fifteenth day produced a repetition of the effect of each of the previous doses (fig. 1).

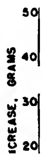


Fig. 1 Effect of single doses of pantothenic acid on the growth of rats depleted of pantothenic acid on a vitamin B complex free diet supplemented with 40 µg. of each, thiamin, riboflavin, and vitamin B_6, 0.5 mg. of nicotinic acid, and 5 mg. of choline chloride per rat per day. At each arrow, 840 µg. of pantothenic acid were given orally.

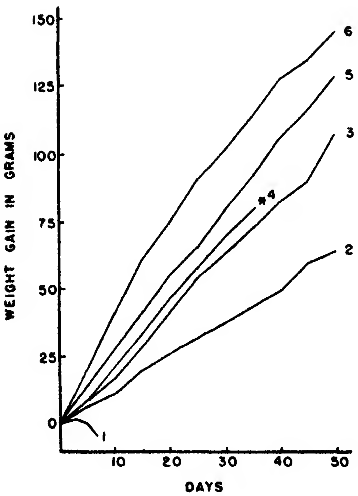
Curative effect following daily feeding

To rats which had reached a stationary weight after a depletion period from 19 to 34 days, daily doses of 12, 25, 50, 100 and 150 µg. of pantothenic acid respectively were administered orally. Groups of ten rats were used for each dose level. Another group received a daily supplement of 0.25 cc. of an aqueous extract of liver [2] containing 150 µg. of pantothenic acid as determined by bacterial assay. This supplement presumably supplies other as yet unidentified factors of the B complex.

In contrast to the administration of single doses, the response to daily feeding was much more uniform (fig. 2). At

[2] Kindly prepared and assayed for its pantothenic acid content by Dr. J. C. Keresztesy.

the 12 μg. level the average weight of the group declined below the starting level and all animals died within 16 days (curve 1). At the 25 μg. level 50% of the animals died within 2 weeks; the remaining rats, however, gained continuously at a rate of 1.25 gm. per day over the 50-day period of the test (curve 2). Of the animals receiving 50, 100 and 150 μg. respectively of pantothenic acid or its equivalent in the form of liver concentrate, 70% survived in each group. One hundred and fifty



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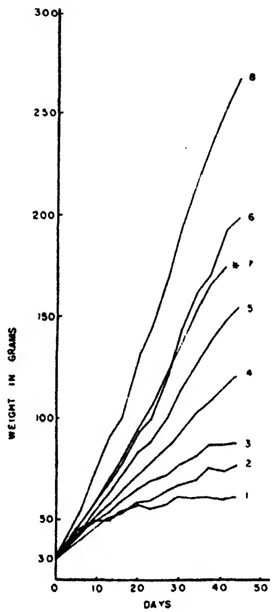

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Fig. 2 Growth response of pantothenic acid deficient rats to daily feeding of graded doses of pantothenic acid. (1) 12 μg.; (2) 25 μg.; (3) 50 μg.; (4) 100 μg.; (5) 150 μg.; (6) 0.25 cc. of a liver concentrate, containing 150 μg. of pantothenic acid. * Animals killed for autopsy.

Fig. 3 Effect of daily feeding of pantothenic acid on the growth of rats maintained on a vitamin B complex free diet supplemented with 40 μg. of each, thiamin, riboflavin, and vitamin B_6, 0.5 mg. of nicotinic acid, and 5 mg. of choline chloride. (1) No pantothenic acid; (2) 5 μg.; (3) 10 μg.; (4) 20 μg.; (5) 40 μg.; (6) 80 μg.; (7) 150 μg.; (8) 10% liver added to the diet containing 150 μg. of pantothenic acid per gram. * Animals killed for autopsy.

micrograms daily produced an average weight gain of 2.5 gm. per day (curve 5). This response was definitely greater than that following daily administration of 50 or 100 μg. (curves 3 and 4). Feeding the liver preparation containing 150 μg. of pantothenic acid per dose caused an average weight gain of 3 gm. per day (curve 6), which exceeds the response to 150 μg. of pantothenic acid. While we agree with Woolley ('40) that the administration of liver produces a greater weight gain than that of pantothenic acid alone, our findings with this latter substance are contrary to his, inasmuch as growth is continuous.

Whereas the effect of single doses was of too short duration to influence the deficiency symptoms significantly, definite changes were observed in the animals receiving pantothenic acid daily over a period of 50 days. At the end of the test only one out of seven animals receiving 150 μg. daily still exhibited excessive nasal secretion and some blood on the whiskers. The fur of all animals in this group was less coarse and rough than at the beginning of the test, but still was lacking the lustre of the fur of rats on complete diets. About the same regression of symptoms was found in the group receiving the liver preparation containing 150 μg. of pantothenic acid. In the groups receiving 25 and 50 μg. daily, no definite improvement in external appearance could be observed during the 50-day period.

Prophylactic test

In this experiment a total of ninety 3-week-old male rats, divided into nine groups of ten each, were placed on the same dietary regimen as is described on page 566. They were further supplemented as follows: One group receiving no pantothenic acid served as a negative control. A second group receiving whole dried beef liver[3] mixed with the diet to an extent of 10% served as a full diet control. The pantothenic acid content of the liver, as determined by the bacterial growth

[3] Kindly prepared and assayed for its pantothenic acid content by Dr. J. C. Keresztesy.

method, was sufficient to supply 150 µg. per 10 gm. of diet. To the remaining groups various doses of pantothenic acid were administered daily orally. One of the groups on 150 µg. daily did not receive nicotinic acid amide. The average weight responses to the daily feeding of graded doses of pantothenic acid are recorded in figure 3. The animals receiving no pantothenic acid reached stationary weight after 30 days on the test diet (curve 1). The group receiving 5 µg. daily likewise ceased to gain about the thirty-seventh day but attained a slightly higher weight than the negative control group (curve 2). Ten micrograms daily were still insufficient to produce continuous growth, the weight becoming almost stationary at 90 gm. (curve 3). Higher doses produced continuous growth in the rats throughout the test period as evidenced by curves 4, 5 and 6 in figure 3. No significant difference was found between the growth responses to 80 and 150 µg. of pantothenic acid daily. The average weight gain per rat per day obtained with these doses was 4 gm. On the other hand, the rats receiving dried liver gained weight at an average of 5.3 gm. per day. The group receiving 150 µg. of pantothenic acid but no nicotinic acid amide grew at a somewhat slower rate than the corresponding group with nicotinic acid amide.

The manifestations noted in the animals receiving no pantothenic acid have already been described. Daily doses of 5 or 10 µg. of pantothenic acid failed to produce any improvement in appearance as compared with the negative control group. Twenty micrograms daily, while sufficient to prevent death and to allow continuous growth throughout the test period, likewise failed to influence appreciably the deficiency symptoms. The animals receiving 40 µg. daily developed a much better coat, definitely less thin and coarse than that of the animals on the lower dose levels of pantothenic acid, but still lacking the smoothness and the lustre of normal fur. In this group no blood-caked whiskers were observed, and only a few animals showed rusty spots on the fur. On the forty-third day of the test, these appearances seemed to be in regression. On a daily dose of 80 µg. of pantothenic acid the animals did not

exhibit any external symptoms except for a short period between the third and fifth weeks, when the coat was somewhat rough and not in as good condition as that of the animals receiving 150 μg. of pantothenic acid or the liver supplement. This manifestation, however, was transient; after the thirty-fifth day the fur of all animals receiving 80 μg. matched that of the animals receiving liver. With the exception of their respective weights, no significant difference could be found at

TABLE 1

Effect of pantothenic acid on the incidence of deficiency symptoms observed in rats maintained on a vitamin B complex free diet supplemented with 40 μg. of each, thiamin, riboflavin, and B_6, 0.5 mg. of nicotinic acid, and 5 mg. choline chloride per rat per day

DAILY DOSE OF P.A. IN MICROGRAMS	PER CENT SURVIVAL AFTER 6 WEEKS	STATIONARY WEIGHT REACHED ON	COARSE THIN, FUR	BLOOD-CAKED WHISKERS	RUSTY SPOTS	PER CENT OF SPECTACLED EYES	PER CENT OF ADRENAL HEMORRHAGES
0	40	30th day	++	++	++	20	40
5	40	37th day	++	++	++	0	20
10	90	50th day	++	++	+	10	10
20	100		++	++	++	0	0
40	100		+	0	+	0	0
80	100		(+)	0	0	0	0
150	100		0	0	0	0	0
150 in liver	100		0	0	0	0	0

the end of the test period in the appearance of the rats receiving 80 or 150 μg. of pantothenic acid and those on the liver supplement. In table 1 an attempt has been made to summarize the incidence of the various symptoms in the different groups.

DISCUSSION

Effect on growth

Since the substance used in our experiments was pantothenic acid obtained by condensation of the pure "natural" lactone and beta-alanine, it seemed advisable to study the effect of each of the constituents of the molecule. Beta-alanine alone administered to groups of ten deficient rats in single doses of 1.8 and 9.3 mg. respectively was without any significant effect,

confirming the findings of El-Sadr et al. ('39), Oleson, Woolley and Elvehjem ('39), and György, Poling and Subbarow ('39). Daily feeding of 0.5 mg. of either beta-alanine, the lactone or both together over a period of 40 days to groups of ten young rats on the basal diet did not increase the growth rate over that of the negative control group. Thus, it is evident that the rat cannot utilize the components of pantothenic acid singly or when mixed together, but needs the intact pantothenic acid molecule. Hence, the effects obtained in our experiments must be attributed to the administration of pantothenic acid per se.

Young rats maintained on the basal diet supplemented with the synthetic crystalline factors, attained a growth rate of 4 gm. per day when fed with 80 μg. of pantothenic acid per day. Larger doses did not increase this figure. However, the addition of 10% of dried liver to the diet (equivalent to feeding 150 μg. of pantothenic acid per 10 gm. of food) produced a growth rate in excess of that found with pantothenic acid alone. These findings demonstrate the important role of pantothenic acid as a growth stimulating substance and at the same time, offer further evidence for the presence of additional growth stimulating factors in liver.

Effects on external symptoms

Of the deficiency symptoms manifested on our diet, almost every one has been previously observed either singly or in combination in animals on diets devoid of the "filtrate factor." It should be kept in mind that the diets used by different investigators varied greatly in the percentage and character of their constituents and supplements, a fact which might exert considerable influence on the time, appearance, incidence and degree of severity of the lesions. Hence, comparisons between our findings and those of other investigators can only be tentative.

The most consistent symptom observed in our experiments was the change in the condition of the fur. György and Eckhardt ('39) record, in some of their deficient animals, an

alopecia extending over the neck and back, but give greater emphasis to the scaliness and inflammation of the skin. Lepkovsky, Jukes and Krause ('36) mention dermatitis; and Chick, Macrae and Worden ('40) emphasize the characteristic change in the fur of animals deficient in "filtrate factor."

Excessive nasal secretion occasionally containing blood was observed in the majority of our animals. "Inflammed nose" was recorded by Lepkovsky, Jukes and Krause ('36) in rats lacking the filtrate factor, and recently Daft and Sebrell ('39) found nose bleed a predominant sign in rats on a B complex diet but supplemented with the crystalline factors.

No mention of blood-caked whiskers, which we observed in the majority of our animals, could be found in the recent literature. Whether this symptom and the rusty spots or "browning" (Lunde and Kringstad, '39) of the hair is caused by the animals rubbing blood from the nose or from sores around the mouth onto the fur and the whiskers is not as yet established.

In agreement with the statement by György and Eckhardt ('39) "spectacled" eyes were found in only a small percentage of our animals. The description given by Lepkovsky, Jukes and Krause ('36) and Lunde and Kringstad ('39) holds good for the signs as observed on our diet, except that no erosions (Oleson, Bird, Elvehjem and Hart, '38; Oleson, Elvehjem and Hart, '39) were found around the eye.

Autopsies on our deficient rats confirmed the findings of Daft and Sebrell ('39) and Nelson ('39) on adrenal cortical hemorrhages.

At the present time it cannot be decided definitely whether all or some of the symptoms are specifically due to lack of pantothenic acid in the diet. Although the administration of pantothenic acid produces an improvement of the external symptoms, chiefly noticeable in the condition of the fur, the response was rather delayed and high doses over a prolonged period of time had to be administered to demonstrate a curative effect. This contrasts sharply with the dramatic improvement of acrodynia lesions following the administration of vitamin B_6.

On the other hand, a close relationship between pantothenic acid and the deficiency syndrome becomes obvious from the results of the prophylactic test, summarized in table 1. None of the manifestations occurred on daily administration of 150 µg. of pantothenic acid, a dose above the requirement for maximal growth. With 80 µg. daily only fleeting signs of fur impairment were observed. Particularly, no adrenal hemorrhages were found with doses which assured continuous growth.

ACKNOWLEDGMENT

The valuable technical assistance of Miss Grace Richards and Miss Evelyn Ruddy is gratefully acknowledged.

SUMMARY[4]

The effect of pantothenic acid prepared from pure "natural" α-hydroxy-β, β-dimethyl-gamma-butyrolactone and β-alanine has been studied on rats maintained on a synthetic diet supplemented with the synthetic crystalline factors of the B complex.

1. Rats maintained on a diet devoid of pantothenic acid cease to grow after 3 to 4 weeks and develop a deficiency syndrome characterized by scant, coarse fur showing rusty spots, inflammation of the nose, and blood-caked whiskers. On autopsy some of the rats show hemorrhages under the skin and into the adrenal cortex.

2. A single dose of 800 µg. or daily feeding of 50 µg. of pantothenic acid produces a marked weight response in deficient rats, but the deficiency symptoms yield only after prolonged administration.

3. In prophylactic tests the maintenance dose for optimal growth is approximately 80 µg. of pantothenic acid per rat per day.

4. Liver concentrates of known pantothenic acid content are superior to equal doses of pantothenic acid alone in their growth-promoting effect.

[4] While this study was in progress, synthetic pantothenic acid (Williams and Major, '40) became available. Using calcium pantothenate we have duplicated all of our findings on the curative effects reported above.

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THE EFFECT OF VITAMINS A AND C ON EXPERIMENTAL HYPERTHYROIDISM [1]

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TWO FIGURES

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Since 1932, numerous reports have appeared in the literature pertaining to the antagonism demonstrated by vitamin A or its precursor, carotene, to thyrotoxicosis (Murlin, '38). During thyrotoxicosis, von Euler and Klussmann ('32) found vitamin A stores of the liver to be depleted, and that carotene would counteract the body weight loss associated with this condition. According to Eufinger and Gottlieb ('35) vitamin A will depress the acceleration of metamorphosis of tadpoles induced by thyroxin. Rappai and Rosenfeld ('35) demonstrated that the hypermetabolism produced by thyroxin injections can be reduced to some extent by vitamin A therapy, but the increased metabolism induced by dinitrophenol, according to the experiments of Logaras and Drummond ('38), will not be affected. Abnormalities of carbohydrate metabolism associated with hyperthyroidism have been reported by David ('38) and by Egelin ('39) to be reduced to a minimum by vitamin A. From the work of Fasold and Heidemann ('33) it seems that the thyroid is necessary in some way for the conversion of carotene to vitamin A. This was evident after they noted that thyroidectomy changed the composition of

[1] This investigation was supported by a grant of the National Research Council's Committee on Endocrinology.

[2] The data in this paper were taken from a thesis presented in partial fulfillment of the requirements for the doctorate of philosophy, University of Rochester, June, 1940.

goat milk from its usually high vitamin A content to one high in carotene. Wohl and Feldman ('39) bear out this relationship in their findings of pathological dark adaptation amongst their cases of hypothyroidism. With increased metabolism, Sure and Buchanan ('37) have shown that there is an increased requirement of vitamin A.

Independently, Demole and Ippen ('35) and Oehme ('36) reported ascorbic acid capable of reducing the hypermetabolism of thyrotoxicosis. Creatinuria of a hyper-thyroid animal or human can be suppressed by ascorbic acid therapy, as shown by Fischer and Oehme ('37) and by v. Plehwe ('38). The experiments of Marine, Baumann and Rosen ('34) as well as those by Schulze and Linnemann ('38) point to the idea that vitamin C is able to inhibit the action of thyrotropic hormone on the thyroid gland. The blood picture presented by the scorbutic guinea pig resembles that of an hyperthyroid animal (Aszódi, '37).

PROCEDURE

Rats used in this investigation were of the Wistar strain. Only one major group of animals of similar age and sex was utilized for the study of the efficacy of ascorbic acid in counteracting the hypermetabolism of hyperthyroidism. Two major groups of rats were used in the vitamin A studies. Each group was subdivided into: (1) control rats which were on the stock diet of commercial dog food,[3] (2) rats receiving only vitamin A or ascorbic acid in addition to the stock diet, (3) animals receiving thyroxin injections, and finally (4) rats receiving vitamin A or ascorbic acid in addition to the thyroxin injections.

Crystalline thyroxin [4] which was dissolved in N/100 NaOH, was injected subcutaneously in the amount of 0.2 mg. per 100 gm. of body weight daily over a period of 6 successive days. The animals were weighed daily and the dose of thyroxin was adjusted accordingly.

[3] Purina dog chow.

[4] Squibb.

The vitamin A concentrate was given orally in the amount of 2000 U.S.P. units/day over a period of 8 days. The animals receiving this concentrate along with the thyroxin injections (6 days), received their vitamin A supplement 2 days previous to the initiation of the thyroxin administration. This permitted the animal to build up its vitamin A stores. Ascorbic acid was injected subcutaneously in the amount of 20 mg. per 100 gm. of body weight per day for 6 successive days. In experiments employing both vitamin C and thyroxin the two were administered over the same length of period.

On the sixth and last day of injection, the basal metabolism was determined on the rats in the post-absorptive condition. The apparatus used for such determinations employed the Haldane principle, moisture, carbon dioxide production and oxygen consumption being determined gravimetrically. This particular form of apparatus was constructed similarly to the one employed by Forbes, Kriss and Miller ('34) and was the same apparatus used by Black ('39). All measurements were made with the temperature of the respiration chamber between 28°C. and 29°C. The length of the experimental period was 6 hours. Basal heat production values are expressed as Calories per square meter of body surface per hour, according to the formula of Diack ('30).

On the seventh day the animal still in the post-absorptive state was sacrificed by a blow on the head, and the liver, kidneys and thyroid removed as quickly as possible. The tissues were sliced with a straight sectioning razor. The respiration of these tissues was determined by means of the Warburg constant volume manometers utilizing the Marsh ('37) type of respiration flask. This type of flask permitted the determination of Q_{O_2}, Q_{CO_2} and consequently the respiratory quotient in the same flask, although appropriate controls were necessary. Both kidney cortex and liver tissue slices were run in triplicate, but only single determinations of thyroid respiration (Q_{O_2}) were made, since the thyroids of rats are quite small. The tissue medium used was a Ringer-

TABLE 1

Basal metabolism data of vitamin studies in experimental thyrotoxicosis

	SEX	AGE	NO. IN GROUP	CAL./HR.	PER CENT CHANGE	CAL./KG./HR.	PER CENT CHANGE	CAL./M^2/HR.	PER CENT CHANGE	R.Q.
		(months)								
Controls	F	6	6	0.894	....	5.009	....	36.9	....	0.73
Controls receiving vitamin C	F	6	4	0.852	—5	4.889	—4	35.9	—3	0.74
Thyroxin treated	F	6	5	1.350	+51	8.173	+63	58.7	+59	0.73
Thyroxin treated + vitamin C	F	6	5	1.180	+32	6.949	+39	49.8	+35	0.73
Controls	M	4	6	1.156	....	4.698	....	38.5	....	0.73
Controls receiving vitamin A	M	4	6	1.156	0	4.710	0	38.6	0	0.73
Thyroxin treated	M	4	6	1.933	+67	9.920	+111	75.2	+95	0.73
Thyroxin treated + vitamin A	M	4	5	1.943	+68	7.789	+66	64.1	+66	0.73
Controls	F	18	6	1.001	....	4.453	....	35.4	....	0.73
Controls receiving vitamin A	F	18	5	1.041	+4	4.893	+9	38.5	+9	0.73
Thyroxin treated	F	18	6	1.652	+65	7.777	+75	60.4	+71	0.73
Thyroxin treated + vitamin A	F	18	6	1.528	+53	7.045	+58	55.5	+57	0.74

TABLE 2

Tissue respiration data of vitamin studies in experimental thyrotoxicosis

	SEX	AGE	NO. IN GROUP	LIVER Q_{O_2}	Per cent change	Q_{CO_2}	Per cent change	R.Q.	KIDNEY Q_{O_2}	Per cent change	Q_{CO_2}	Per cent change	R.Q.	THYROID Q_{O_2}	Per cent change
		(*months*)													
Controls	F	6	6	7.05	....	3.65	....	0.52	17.3	....	14.5	....	0.84	4.14	....
Controls + vitamin C	F	6	4	8.11	+15	3.91	+7	0.48	19.4	+12	16.1	+11	0.83	5.13	+24
Thyroxin treated	F	6	6	8.46	+20	4.12	+13	0.49	19.0	+10	15.8	+9	0.83	2.60	—37
Thyroxin treated + vitamin C	F	6	5	8.72	+24	4.06	+11	0.46	20.1	+16	17.0	+17	0.84	2.70	—35
Controls	M	4	6	7.07	....	3.94	....	0.56	17.2	....	13.9	....	0.81	5.35	....
Controls + vitamin A	M	4	6	7.52	+6	3.79	—4	0.50	18.3	+6	15.2	+9	0.83	4.83	—10
Thyroxin treated	M	4	6	8.69	+23	4.38	+11	0.51	19.1	+17	15.4	+11	0.81	3.33	—38
Thyroxin treated + vitamin A	M	4	5	9.62	+36	4.63	+18	0.49	20.5	+19	16.8	+21	0.82	2.75	—49
Controls	F	18	6	7.01	....	3.35	....	0.47	17.0	....	13.7	....	0.81	3.74	....
Controls + vitamin A	F	18	5	7.63	+9	3.75	+12	0.49	17.0	0	13.2	—3	0.78	3.37	—10
Thyroxin treated	F	18	6	9.26	+32	4.49	+34	0.48	20.6	+21	16.8	+23	0.82	2.63	—30
Thyroxin treated + vitamin A	F	18	6	9.38	+34	4.46	+32	0.47	19.9	+17	15.7	+15	0.79	2.75	—26

phosphate-glucose solution, the gas space in the respiration flask being filled with air (Belasco, '40).

RESULTS

Summaries of the basal metabolism and tissue respiration data are shown in tables 1 and 2 and in figures 1 and 2. Vitamin C injections alone did not alter the basal heat production of the 6-month-old females. When ascorbic acid was

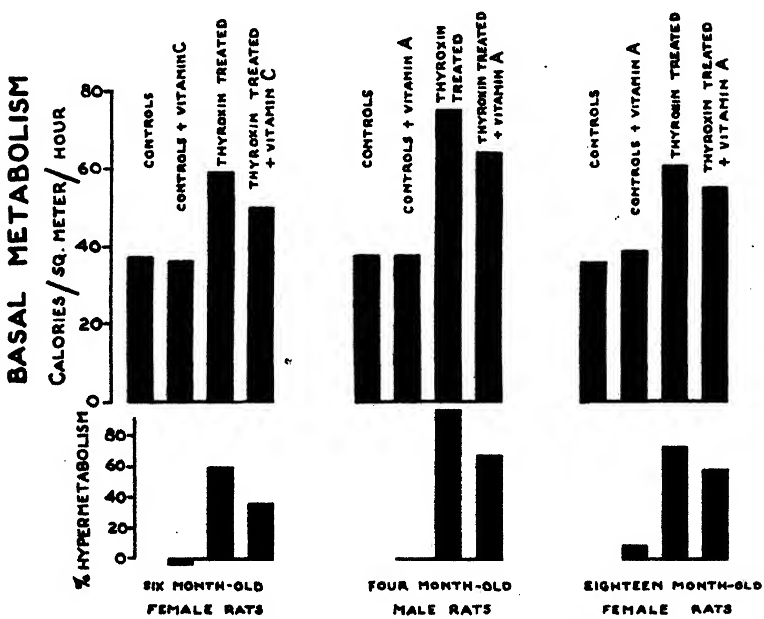

Fig. 1 The effects on the energy metabolism of rats of vitamins A and C administered with and without thyroxin. Note the effect of the vitamins in decreasing the hypermetabolism due to thyroxin.

given in conjunction with thyroxin injections, the resulting hypermetabolism was much less than the increased metabolism due to thyroxin alone. Thyroxin, by itself, produced an increase in heat production of 59%; this hypermetabolism was reduced to only 35% when ascorbic acid was given simultaneously with thyroxin.

From the point of view of the tissue metabolism data, this antagonism was not evident. Ascorbic acid administration alone caused increased liver, kidney and thyroid respiration. When thyroxin was injected simultaneously with vitamin C, the metabolism of liver and kidney tissue was even greater than would have resulted, if thyroxin alone were injected. Vitamin C in no way alleviated the depressed thyroid respiration that was produced by thyroxin treatment.

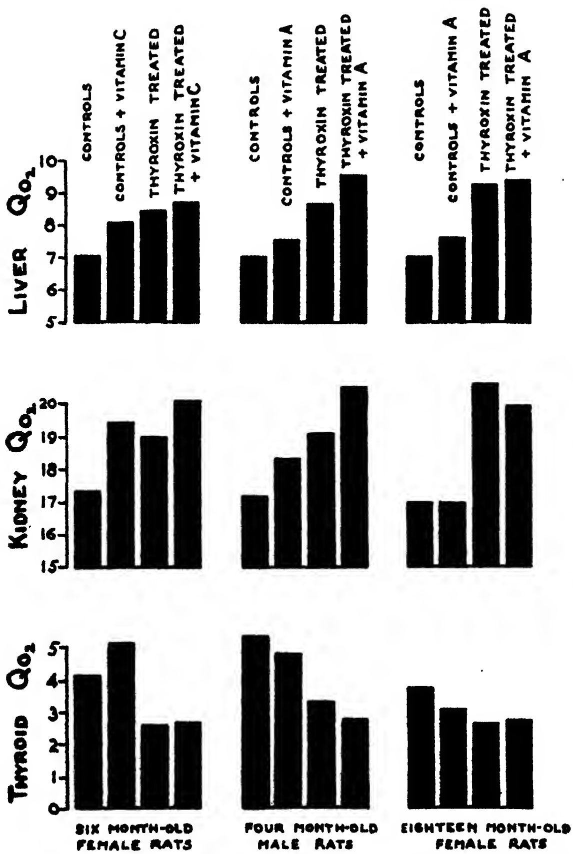

Fig. 2 The effect on the respiration of liver, kidney and thyroid of vitamins A and C administered with and without thyroxin.

Feeding the vitamin A concentrate alone did not alter the basal metabolism significantly. The 4-month-old males receiving this vitamin concentrate had exactly the same basal heat production as their controls. On the other hand, the metabolism of 18-month-old females seemed to be increased to the extent of 9% by vitamin A ingestion. The variation cannot be considered as significant at present because of the small number of animals used. The hypermetabolism of the 4-month-old males receiving thyroxin was 95% above normal basal metabolism. Treatment of thyrotoxicosis with vitamin A resulted in an increased metabolism of only 66%. The hypermetabolism of the thyroxin-treated 18-month-old female rats was reduced from +71 to +57 by the feeding of vitamin A concentrate.

The response of liver, kidney and thyroid respiration to vitamin A resembled the tissue respiration of thyroxin-treated animals, but to a lesser degree; that is to say, vitamin A stimulated hepatic and renal tissues to higher metabolic levels in the 4-month-old males, while it depressed the metabolism of thyroid tissue. In the aged females the vitamin stimulated only the liver tissue. Comparing the metabolism of tissues of rats receiving thyroxin and of those receiving the vitamin A concentrate along with thyroxin, no antagonism was evident at all. In fact, the liver metabolism was increased and the thyroid respiration decreased still further in the 4-month-old males. In the 18-month-old females this additive effect was very slight, and present only with liver.

DISCUSSION

There was a definite antagonism between both vitamins and thyroxin as evidenced by the lowering of the hypermetabolism of animals receiving either of the vitamins in conjunction with thyroxin. The tissue respiration data give no indication of this antagonism with either vitamin. Both vitamin A and ascorbic acid increased liver and kidney tissue respiration in the young rats. The point of difference between the two vitamins, in this regard, is that vitamin A depressed thyroid

oxygen uptake to the extent of 10%, while vitamin C increased the metabolism of the same gland 24%. These vitamins given in conjunction with thyroxin either did not affect the oxygen consumption of liver and kidney tissue derived from the thyrotoxic animal, or they increased the respiration of these tissues still further. Although the vitamin A concentrate alone reduced the thyroid oxygen consumption of thyroid tissue, it produced an enlargement of the same gland. This probably signifies that vitamin A in some way affects the pituitary or the thyroid gland itself, to cause a cessation of colloid excretion, thus producing a storage of the colloid in the follicles of the gland.

The effect of vitamin C on tissue metabolism in producing increased oxygen consumption is odd in the light of the finding of Stotz et al. ('37) that the liver respiration of scorbutic animals is higher than that of normals. Thus it seems that an excess or a deficiency of ascorbic acid will produce similar results. However, it is plausible to suppose that conditions leading up to increased respiration are not the same in both instances. In the scorbutic animal there is a deficiency of a hydrogen acceptor. In an attempt of the body to maintain its normal level of metabolism, the pathways of oxidation are re-routed, the change leading to less efficient metabolism with consequent increased oxygen consumption. From the other side, it is possible that a relative increase in a dehydrogenase, like ascorbic acid, might tend to bring about an increased oxygen consumption of normal tissues. Cohen and Gerard ('37) are of the opinion that the increased brain tissue metabolism caused by hyperthyroidism is associated with a relative increase of dehydrogenase concentration in the cells as compared to the oxidase system.

In relation to the stimulating effect of vitamin A on liver respiration, Ruddy ('39) recently observed that the liver tissue of vitamin A-deficient rats has an abnormally low oxygen consumption; but upon giving the animal larger amounts of vitamin, liver metabolism rose back to normal and even to a slightly higher level. No comment was made as to why vitamin

A would tend to increase tissue respiration. Heart tissue of a vitamin A deficient rat is known to have a low oxygen consumption. The increased tissue respiration of liver at least, and of kidney in the young male, produced by vitamin A ingestion is large enough to be reckoned with, for all results were obtained in triplicate. It appears that liver and kidney tissue in an effort to catabolize the excess amounts of vitamin A must necessarily have a higher metabolism.

No doubt there is an increased requirement of vitamin A and vitamin C in hyperthyroidism. If these increased requirements are not supplied, thyrotoxicosis becomes the more evident, thus presenting a picture of a more severe degree of hyperthyroidism than is produced by the thyroxin per se. Increasing the vitamin intake under such conditions therefore gives one the idea of a reduction in the degree of hyperthyroidism, while in actuality it may be only the removal of the deleterious effects of vitamin deficiency in this state of toxicity. However, vitamin A in some way antagonizes the active thyroid principle, possibly inhibiting thyroid function, as is evidenced by its ability to depress thyroid tissue respiration, but not that of liver or kidney. Possibly vitamin A, by virtue of its double bonds, is able to take up the available iodine in body tissues, and as an organic iodine compound to antagonize hyperthyroidism as do inorganic or organic iodine compounds, exclusive of thyroxin or thyroglobulin. Either by acting on the anterior pituitary gland or on the thyroid directly, this iodinated vitamin A may affect the storage of colloid and consequently cause lowered oxygen uptake of the thyroid. This antagonism will have to be studied more closely in the future to uncover the true mechanism.

SUMMARY

The basal metabolism and liver, kidney and thyroid tissue respirations of rats were studied to determine whether vitamin A and ascorbic acid could be shown to affect thyrotoxic tissue respiration as they do thyrotoxic basal metabolism. The effect of the vitamins alone was also studied.

Vitamin A and ascorbic acid did reduce the hypermetabolism associated with hyperthyroidism to some extent. Only in one group of rats, the 18-month-old females, did vitamin A raise the metabolism slightly. In the other groups neither vitamin produced any alteration of normal basal heat production.

Both ascorbic acid and vitamin A increased the respiratory rates of liver and of kidney cortical tissue. Ascorbic acid increased thyroid tissue respiration, while vitamin A depressed it. Increased liver and kidney tissue metabolism associated with hyperthyroidism was not altered by vitamins A or C. The depressed thyroid respiration, as a result of thyroxin administration, was not alleviated either by vitamin A or ascorbic acid. In fact, there were instances where the effect was additive, i.e., of the hormone plus the vitamin.

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THE INFLUENCE OF EXERCISE ON THE GROWING RAT IN THE PRESENCE AND ABSENCE OF VITAMIN B_1[1]

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FIVE FIGURES

(Received for publication August 12, 1940)

In a former communication from this laboratory (Guerrant, Dutcher and Chornock, '39) it was shown that when carefully selected groups of young rats were used as experimental subjects under comparable conditions of experimentation, less food was consumed, smaller increases in body weight were made and less severe symptoms of vitamin A deficiency developed when the animals were forced to exercise than when permitted to exercise voluntarily or when confined in the usual type of cage. It was shown that, although animals which were forced to exercise exhibited the greatest efficiency of food utilization, they voided the greatest number of fecal pellets, indicating a beneficial effect of exercise on intestinal motility. Furthermore, it was shown that animals, which were exercised voluntarily, and which received daily allotments of vitamin A, were more active physically than were the litter mates which did not receive the vitamin supplement.

The investigation reported at this time concerns itself with similar studies regarding the effect of exercise on vitamin B_1 requirement.

[1] Authorized for publication on July 31, 1940, as paper no. 982 in the Journal Series of the Pennsylvania Agricultural Experiment Station. Presented before the division of biological chemistry at the Cincinnati meeting of the American Chemical Society, April 10, 1940.

EXPERIMENTAL

The present investigation consisted essentially of a comparison of the physiological activity and the growth responses of comparable groups of young rats while being maintained under identical conditions, with the exception of the amount of exercise permitted by the type of caging employed and of the vitamin B_1 intake. The experimental equipment was identical with that used in the earlier experiments. With the exception of the following details, the experimental procedure was also similar to that used in the previous investigation.

Healthy rats, 23 to 26 days of age and weighing approximately 52 gm., were used as the experimental subjects. All animals were fed, ad libitum, a vitamin B_1 deficient basal diet which had the following percentage composition: purified casein 18, salt mixture 4, cellu flour 2, sucrose 71, filtered butterfat 3 and cod liver oil 2. Each animal received, daily, 10 μg. of riboflavin and the equivalent of 100 mg. of rice polish concentrate[2] as supplements to the basal diet. Each set of animals was divided into two groups. The animals of one group received the unsupplemented vitamin B_1 deficient diet until they were depleted of their body reserves of vitamin B_1. With the development of the paralytic symptoms characteristic of vitamin B_1 deficiency, each animal of this group was fed 6 μg. of vitamin B_1 daily for a period of 35 days. The second group of animals served as positive controls and, in consequence, each animal received 6 μg. of vitamin B_1 daily throughout the experimental period.

DATA

The data reported at this time have been reduced to group averages and are given in figures 1 to 5 inclusive. In making these tabulations only data from those animals which survived the entire experimental period have been included.

[2] Obtained from The Borden Company, Bainbridge, New York. Two hundred grams of this product were dissolved in 750 ml. of distilled water, autoclaved for 4 hours at 15 pounds pressure, cooled, filtered and made up to 1 liter.

DISCUSSION

It was observed that animals in the "forced exercise" group were unable to undergo the desired 1000 cage revolutions daily, during the first few days of the experiment, without becoming exhausted. As a result, the exercise requirement for these animals was less than 1000 revolutions per day, during this period. The number of revolutions, daily, was gradually increased until all animals were meeting the specified exercise requirement of 1000 revolutions per day, by the end of the first week.

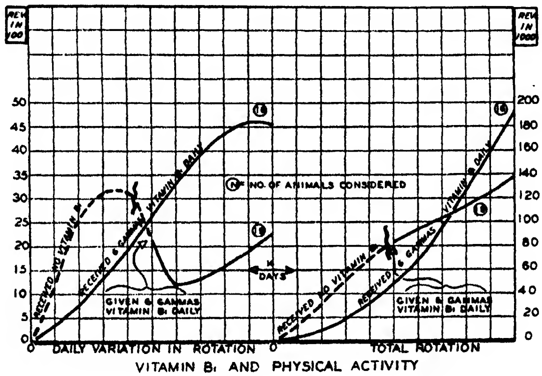

Fig. 1 The mean variation in physical activity of two groups of young rats maintained under comparable conditions in all respects except as to their vitamin B_1 intake.

In the course of these studies a rather significant observation was made in that those animals of the voluntary exercise groups which were depleted of their vitamin B_1 reserve were found to be more active physically, during the first few weeks of the depletion period, than comparable animals, similarly caged, but receiving 6 μg. of vitamin B_1 daily (fig. 1). During the first day or so of the experiment all animals of the voluntary exercise group deported themselves similarly. By the end of the first week, however, it was noted that the animals which were not receiving the vitamin supplement were rotating the wheels of their respective cages more frequently than

were the animals which received the vitamin supplement. This condition became even more noticeable as the experiment progressed. By the twenty-first day of the experiment the animals undergoing depletion were making twice as many revolutions as litter mates receiving thiamin, and approximately three times as many revolutions as the animals of the forced exercise group. However, as the onset of the symptoms of vitamin B_1 deficiency became more pronounced, these animals became progressively less active as paralytic symptoms developed. When the animals reached the stage of depletion where physical excitation resulted in spastic paralysis, they were fed the vitamin supplement. In most instances, it was surprising to observe that such animals continued to become even less active for several days after the supplementary feeding was begun. Usually (about the fourth day of the supplementary feeding of 6 μg. of vitamin B_1 daily), these animals began to regain some of their inclination to exercise and continued to do so until the end of the experiment. However, at the end of the experiment, the average daily activity of the rejuvenated animals was considerably less than the daily activity of similar animals which had received the vitamin from the beginning of the experiment. A possible explanation for this may be found in the relationship of vitamin intake to the weight of the experimental animals. This observation would appear to bear some significance in any attempt to estimate the optimal requirement of the rat for vitamin B_1. In this regard, it appears from the data presented in figure 1, that an intake of 6 μg. of vitamin B_1 is insufficient to maintain the growing rat (50–120 gm.) in a state of nervous stability and that this inadequacy becomes even more pronounced as the weight of the rat increases. Therefore, if such is the case, it is to be expected that the smaller animals receiving 6 μg. of vitamin B_1 daily will be found to be more stable physically than larger animals receiving similar amounts of the vitamin. A further suggestion that the quantity of vitamin B_1 used in these studies (6 μg. daily) was not adequate to maintain the growing rat in a state of nervous equilibrium is found in the fact that the animals used in these studies were far more active physi-

cally than were the animals used in our previous studies where a basal diet containing 8% of dried yeast was fed.

An inspection of the data presented in figures 2 and 3 reveals that exercise, under the conditions in which it was employed in these studies, had a measurable effect on the growth rate of young rats irrespective of whether or not the animals were receiving the vitamin B_1 supplement. Those animals of the voluntary exercise group, which were most active

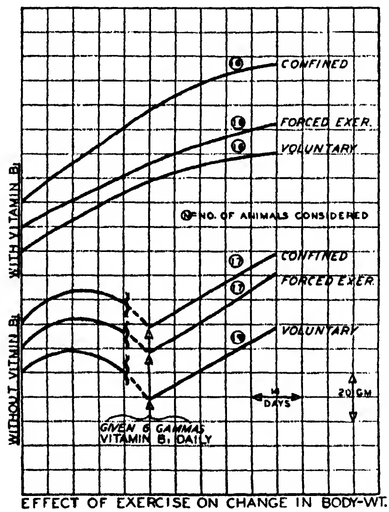

Fig. 2 The effect of different amounts of exercise on the growth rate of young rats while being maintained under comparable dietary conditions except as to their vitamin B_1 intake.

in rotating their cage wheels made smaller increases in body weight than did the less active animals. This observation was most striking during the depletion period, where the animals of the voluntary exercise group showed a smaller initial increase in body weight (8 gm.) and in turn lost a greater percentage of this weight (32%) before developing the paralytic symptoms. In fact the mean weight of the animals of this group at the end of the depletion period was 41 gm., or 11 gm. less than their initial weight (when placed on experiment). The maxi-

mum gains in weight for the three groups of animals (voluntary exercise, forced exercise, and confined) during the depletion period were 8, 13, and 14 gm. respectively, while the subsequent losses in weight for the same groups of animals were 20, 16, and 15 gm. respectively. The mean increase in body weight following the supplementary feeding of vitamin B_1 was found to be quite comparable for the three conditions of exercise. These values, when given in the above order of groups, were 29, 33, and 30 gm. respectively. In this connection, it should be borne in mind that the activity of the animals

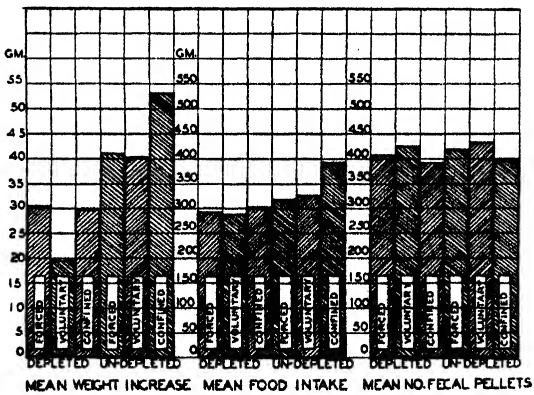

Fig. 3 The relative effects of exercise on the mean body weight increase, the mean food intake and the mean number of fecal pellets voided by young rats while receiving diets identical in all respects except in their vitamin B_1 content.

of the voluntary exercise group, during this time, was considerably less than during the depletion period, and probably more comparable to the activity of the forced exercise animals. In the instance of the animals which received the vitamin supplement from the beginning of the experiment, those of the confined group grew at a considerably faster rate than did the animals of either of the other two groups (voluntary and forced). While it is possible that the nature or the composition of the body weight increments produced under the different conditions of exercising may have been quite different, no data are at hand to substantiate this view. However, later studies

on the chemical composition of body tissues may shed further light on this problem.

From the standpoint of food consumption, the various degrees of exercise did not appear to be an important consideration (fig. 3). Although under both conditions of vitamin supplementation, the animals of the confined groups consumed the greatest amounts of food during the experimental period, the relationship of food intake to body activity is not clearly defined in all instances. It is evident from these data that the matter of growth is a complex phenomenon which is influenced by a number of factors, among which are food intake and exercise.

A similar consideration of the fecal output of the various groups of animals (fig. 3) reveals that there was no great variation in the number of fecal pellets voided by the respective groups of animals during the entire experimental period. However, it seems significant that in both instances the animals of the voluntary exercise groups voided the greatest number of fecal pellets while the animals of the confined group voided the smallest number of fecal pellets. A tabulation of the data regarding the effect of exercise on the weight of the fecal matter, the percentage of fecal matter (relative proportion of food excreted as fecal matter) and the relative sizes of the fecal particles (fig. 4) reveals some significant differences between the animals of the three experimental groups in these respects.

The animals of the confined groups produced the greatest weight of fecal matter, the greatest percentage of fecal matter and the largest fecal pellets (as indicated by weight), while the animals which exercised most strenuously (voluntary exercise group) produced the least fecal matter, the smallest percentage of fecal matter and the smallest fecal pellets. While some of these differences may not be significant, the data clearly indicate a beneficial effect of exercise on fecal elimination. Another interesting observation relates to the fact that under all conditions of exercise, those animals which received vitamin B_1 utilized their food more efficiently (lower

percentage of fecal matter) than did similar animals not receiving the vitamin supplement.

The data given in figure 5 demonstrate the effect of exercise on the average time required for the animals of the various

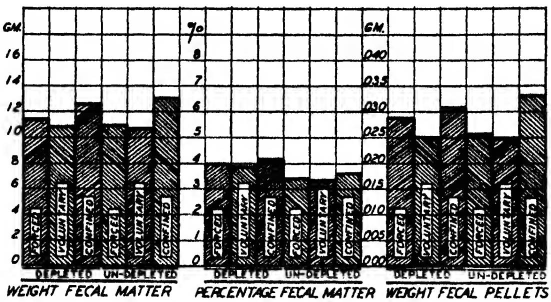

Fig. 4 The effect of exercise on total weight of fecal matter, percentage of fecal matter and mean weight of fecal pellets voided by young rats while receiving diets identical in all respects except as to the amount of the vitamin B_1 supplement.

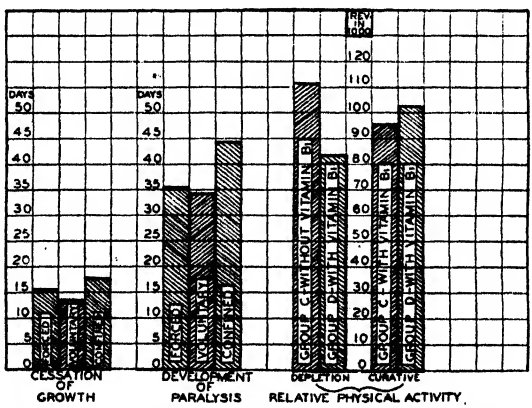

Fig. 5 The effect of exercise on the time required for the cessation of growth and for the development of the paralytic symptoms among groups of young rats receiving a diet deficient in vitamin B_1. The figure also shows the relative activity (as indicated by the rotation of the cage wheel) of two comparable groups of young rats, of which the animals of one group (group D) received daily 6 μg. of vitamin B_1 throughout the experimental period, while the animals of the other group (group C) received no added vitamin B_1 during the depletion period but received daily 6 μg. of this vitamin during the last 35 days of the experimental period.

groups, undergoing depletion, to cease growing. It will be noted that the animals of the voluntary exercise group ceased growing after about 13.5 days on the deficient diet, while the animals of the forced exercise and of the confined groups required 16 and 17.6 days, respectively. Corresponding data relative to the average time required for the development of the paralytic symptoms were equally significant. The animals of the voluntary exercise group manifested the characteristic paralytic symptoms on the thirty-fourth day of the depletion period, while the animals of the forced exercised and of the confined groups required 36 and 44 days respectively. These findings clearly indicate that young rats undergoing vigorous exercise have somewhat greater requirements for vitamin B_1 than do similar animals which undergo little or no exercise. This is in agreement with the data obtained by Cowgill and co-workers ('31) in their studies with dogs but is not in agreement with our previous findings regarding the influence of exercise on the vitamin A requirement of the growing rat. However, it seems highly probable that the latter variation may be due to differences in the physiological function of the two vitamins. It appears reasonable to postulate that the symptoms of vitamin A deficiency are accentuated by the accumulation of toxic substances in the body of the experimental animal due to poor elimination. Therefore, any factor, such as exercise, which increases elimination may bring about a delay in the development of such symptoms. On the other hand, vitamin B_1 which is known to play an important role in the complicated phenomena associated with cellular oxidation and muscle contraction may be required in increased amounts during periods of vigorous exercise. In fact the data obtained in these studies indicate that the vitamin B_1 requirement of the rat is more dependent on the rate of metabolism than it is on the caloric intake.

SUMMARY

Experiments conducted with carefully selected groups of young rats as experimental subjects have yielded data which demonstrate that the vitamin B_1 requirement of these animals,

as indicated by the time required for the cessation of growth and for the development of the paralytic symptoms, is increased by physical exercise. These findings are not in agreement with previous findings concerning the influence of exercise on the vitamin A requirement of the growing rat. A possible explanation of this variation may be found in the differences in the physiological function or functions of the two vitamins.

In the instance of the animals receiving no vitamin B_1, increased exercise resulted in less food consumption, less growth, early development of paralytic symptoms and the elimination of a greater number of fecal particles. With the animals receiving 6 μg. of vitamin B_1 daily, increased exercise resulted in less growth and in the elimination of a greater number of fecal pellets. During the first 4 weeks of the test period, those animals which were permitted to exercise voluntarily were more active physically while not receiving vitamin B_1 than were comparable animals, similarly caged, but receiving vitamin B_1. However, during the latter stages of vitamin B_1 depletion, physical activity among these animals declined to a minimum of about one-third of its previous value. This decline in physical activity continued for approximately 6 days after the administration of daily dosages of 6 μg. of vitamin B_1. There was a steady resumption of activity by these animals during the last 4 weeks of the experimental period, as the result of the administration of the vitamin. Definite nervous instability resulting from vitamin B_1 deficiency was indicated, during the early part of the depletion period, by the increased tendency of the animals to exercise.

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STUDIES ON A DERMATITIS IN CHICKS DISTINCT FROM PANTOTHENIC ACID DEFICIENCY [1]

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ONE FIGURE

Extensive work in several laboratories (Woolley et al., '38, '39; Jukes, '39; Williams and Major, '40) has established the essential nature and role of pantothenic acid in the prevention and cure of chick dermatitis, and generally "chick dermatitis" is used to refer to pantothenic acid deficiency. However, it has been known for several years that similar lesions may be produced in chicks and other animals by the inclusion of raw egg white in the ration. Lease and Parsons ('34) and others demonstrated that the chick antidermatitis factor was not curative for this condition and further studies have supported this conclusion. Although rats have been used to a large extent in the investigations upon the anti-egg white injury factor, termed vitamin H by Gyorgy ('37), there is no reason to believe that the results obtained do not apply to chicks. The properties, distribution, and concentration of

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We are indebted to Merck and Company, Rahway, New Jersey, for generous supplies of thiamin and vitamin B_6; to the Abbott Laboratories, North Chicago, for haliver oil; to the Pabst Brewing Company, Milwaukee, for yeast; to Wilson Laboratories, Chicago, for liver extract; and to Allied Mills Inc., Peoria, Illinois, for soybean oil.

vitamin H have been investigated most extensively by Gyorgy and associates (Gyorgy, '39; Birch and Gyorgy, '39; Gyorgy, Kuhn and Lederer, '39). Recently Gyorgy, Melville, Burk and du Vigneaud ('40) have pointed to the marked similarity of vitamin H and biotin, the bacterial growth factor, and state that no serious difficulty is encountered in correlating the distribution and properties of these factors. Potent concentrates of vitamin H were also said to be high in biotin.

During studies on purified rations for chicks in this laboratory we have observed many cases of dermatitis upon rations apparently adequate in pantothenic acid. In this paper we wish to report studies on this condition. The preventive and curative factor is similar to vitamin H and biotin in distribution and properties and potent vitamin H concentrates have also been found to be active.

EXPERIMENTAL

The rations used in these studies have varied in the kind and amount of liver extract used. Essentially they had the following percentage composition: purified casein 18, salt mixture 5, defatted cartilage 15, soybean oil 5, liver extract (fraction D)[2] 3 to 8, and dextrin 49 to 54. Thiamin and vitamin B_6 were added at the rate of 2 mg. per kilo and a vitamin A and D concentrate was fed separately. In some cases a yeast eluate was added to supply a growth factor necessary in addition to alcoholic liver extracts (Hegsted et al., '40 a) which is presumably factor U (Stokstad and Manning, '38). The liver extracts used have been shown to supply adequate pantothenic acid and flavin at a 2% level and cartilage supplies the cartilage growth factor (Hegsted et al., '40 b).

Day-old White Leghorn chicks were used throughout. They were placed on raised screens in small heated brooders with the experimental ration and water supplied ad libitum. Weighings were made weekly and the chicks were examined frequently for lesions on the feet and around the beak.

[2] Wilson Laboratories.

RESULTS

Although some variation has been found in the time necessary for the depletion of different groups of chicks, incipient lesions similar to those seen in egg white toxicity usually appear in about 3 weeks. The bottoms of the feet become rough and calloused and may be severely affected before mandibular lesions are evident. As the syndrome progressed the entire bottom of the foot becomes encrusted and hemorrhagic cracks appear (fig. 1). The toes may become necrotic and slough off

Fig. 1 Dermatitis produced on a ration adequate in pantothenic acid. Note the severity of the foot lesions.

but the top of the foot and leg usually show only a dry scaliness. The mandibular lesions which first appear in the corners of the mouth spread to include the area around the beak, and the eyelids become swollen and stick together. In contradistinction to these symptoms the lesions in pantothenic acid deficiency are first evident in the corners of the mouth and seldom if ever do the lesions on the feet become as severe as in the new syndrome described.

The preventive effect of various supplements is shown in table 1. The average weight at 4 weeks of age is also given although antidermatitis potency is not paralleled by growth effect upon this ration. When 8% of liver extract is included in the ration large amounts of pantothenic acid are supplied.

TABLE 1

The activity of various supplements in the prevention of dermatitis

SUPPLEMENT TO BASAL RATION	NUMBER OF CHICKS	AVERAGE WEIGHT AT 4 WEEKS	NUMBER DEAD AT 4 WEEKS	DERMATITIS		
				None	Slight	Severe
		gm.				
None	29	132	5[1]		5	21
2 mg. pantothenic acid per 100 gm.	6	128	1			5
5% commercial yeast extract	6	138				6
5% Vitab	11	152		1	2	8
10% Vitab	6	137			3	3
3% molasses	6	137				6
6% molasses	6	130			4	2
12% molasses	6	142		2	3	1
20% heated middlings	5	161	1	1		3
45% heated yellow corn	11	152		4	6	1
10% fishmeal (replacing casein)	6	159		1	2	3
49% polished rice	6	177		2	2	2
2% kidney residue	6	178		6		
3% kidney residue	12	159	2	10		
6% kidney residue	6	170		6		
MeOH ext. of papain digest of kidney residue = 5%	6	132	1	5		
3% liver residue	6	153		6		
5% brewers' yeast	11	182	1	10		
5% heated brewers' yeast	6	169		5	1	
10% yeast residue	11	171	3	8		
2% kidney residue + 15% additional cartilage	6	172		6		
22% additional casein (no cartilage)	6	96			5	1

[1] Two of these died late enough in the 4-week period to be examined for dermatitis.

However, to rule out definitely its effect the ration was further supplemented with a pantothenic acid concentrate. No growth response or prevention of dermatitis was observed. Yeast extract, a commercial rice polish concentrate,[3] and molasses were relatively poor sources of the antidermatitis factor.

[3] Vitab.

Heated middlings and yellow corn, fish meal and polished rice were only partially effective at rather high levels. Complete protection was obtained with 2% of kidney residue prepared by extracting dried defatted kidney with large volumes of hot water and 50% alcohol. The factor is obviously very difficult to extract from kidney but proved to be water and methanol soluble after digestion of the kidney residue with papain. Liver residue and brewers' yeast were also potent sources of the factor. Five per cent of brewers' yeast heated to destroy pantothenic acid allowed only one slight case, and yeast residue after 50% methanol extraction was also effective.

In order to eliminate the possibility that cartilage was acting in a manner similar to egg white, a low level of kidney residue was added together with additional cartilage, but no dermatitis occurred. Also, in one group the cartilage was completely eliminated and replaced by casein. Growth was poor but all of the chicks developed dermatitis which was later cured by kidney residue.

Curative tests have also been used successfully. Since spontaneous cures have not been noted this procedure may be more satisfactory than the preventive method. The protocols (table 2) show that beef spleen and round were less potent than either kidney or yeast. Considerable variation may be noted and 10% of these materials produced only slight improvement in 3 weeks in two of the chicks. Potent sources, such as 6% of brewers' yeast, or kidney, or liver residue, produced dramatic responses; severely affected chicks were cured in less than 2 weeks. A water soluble preparation prepared by autoclaving liver residue for 1 hour with 15% sulfuric acid was also active.

Potent preparations of vitamin H were supplied by Dr. du Vigneaud.[4] Preparation II contained 200 rat units of vitamin H (Gyorgy, '39) per milligram while preparation III was less pure and contained 25 units per milligram. These were injected twice weekly to supply the levels indicated in table 2. As can be seen, the highest level produced complete cures in 3 weeks and lower levels were nearly as effective.

[4] We wish to express our appreciation to Dr. V. du Vigneaud, Cornell Medical School, New York, for sending us the concentrates of vitamin H.

TABLE 2

Curative effect of various supplements

SUPPLEMENT TO BASAL RATION	CHICK NO.	AGE AT START	DERMATITIS SCORE AT [1]		
			1 week	2 weeks	3 weeks
		weeks			
None	9844	4	3	3	3
None	9845	4	3	3	3
None	9846*	4	2	3	3
5% dried beef spleen	547	5	3	3	1
5% dried beef spleen	537	5	2	1	1
10% dried beef spleen	539*	5	2	1	?
10% dried beef spleen	545	5	3	3	2
5% dried beef round	541	5	2	1	?
5% dried beef round	546*	5	2	1	1
10% dried beef round	540	5	3	3	2
10% dried beef round	550	5	3	3	1
6% liver residue	551	6	3	?	
6% liver residue	560	6	3	0	
15% H_2SO_4 ext. = 5% liver residue	617	4	3	2	?
15% H_2SO_4 ext. = 5% liver residue	603	4	3	?	0
3% brewers' yeast I	544	5	3	2	1
3% brewers' yeast I	552	5	3	3	1
3% brewers' yeast II	558	5	3	2	1
6% brewers' yeast I	549*	5	1	0	0
6% brewers' yeast I	555	5	2	1	0
3% kidney residue	843	4	2	1	0
3% kidney residue	847	4	3	2	0
3% kidney residue	848	4	3	2	?
6% kidney residue	559	3	2	0	
MeOH ext. of papain digest = 1 gm. kidney/week, injected	842	4	2	0	
(Prep. II) 200 units of vitamin H/week, injected [2]	601	4	3	?	0
(Prep. III) 200 units of vitamin H/week, injected	607	4	2	?	0
(Prep. II) 100 units of vitamin H/week, injected	602	4	3	2	?
(Prep. III) 100 units of vitamin H/week, injected	611	4	3	1	0
(Prep. II) 50 units of vitamin H/week, injected	610	4	2	?	0
(Prep. III) 50 units of vitamin H/week, injected	608	4	3	1	?

[1] Chicks marked with asterisk had a dermatitis score of 2 at the start; all others scored 3. A dermatitis score of 3 indicates severe dermatitis on both feet and beak. Smaller numbers indicate less severe cases. Doubtful cases are marked by ? and 0 indicates complete freedom from lesions.

[2] Preparations II and III were assayed for biotin by the bacteriological method by J. O. Lampen and W. H. Peterson. Preparation II contained 5 μg. per milligram and preparation III 0.56 μg. per milligram.

DISCUSSION

The inactivity of liver extract or pantothenic acid preparations, the severity of the foot lesions, and the properties of the active factor clearly differentiate this condition from chick dermatitis caused by lack of pantothenic acid. On the other hand, the similarity of the factor to biotin and vitamin H is indicated by distribution, resistance to extraction, liberation by papain or acid hydrolysis, and stability to heat and strong acid hydrolysis. Finally the activity of potent vitamin H concentrates suggests the similarity of these factors. It should be emphasized that chicks have been cured in 3 weeks by injection of approximately 35 µg. of solids per day. This is a much higher level than rats require but we have no indication that this is a minimum level. Also our chicks weigh from 150 to 250 gm. and we have used a 3-week curative period whereas a 4-week period is used in the rat assay (Gyorgy, '39). Lease ('37) has indicated that chicks require higher levels of vitamin H than rats on rations containing egg white. Thus it may not be surprising that chicks require higher levels than rats, and this may explain why no deficiency of vitamin H has been observed in rats unless egg white is included even though the rations are more highly purified than those we are using for chicks.

It is probable that upon our ration a true deficiency exists since raising the level of cartilage apparently does not increase the requirement and the deficiency has been produced with casein as the sole source of protein. However the possibility remains that all proteins may have the effect of egg white to some extent and that this would only be evident when purified rations low in the protective factor are used.

Although fishmeal, polished rice, middlings, etc., are not potent sources of the factor, they probably contain a sufficient amount of it to explain the failure of other investigators to encounter this condition. Vitamin supplements prepared by autolysis or hydrolysis of liver or yeast may also carry the active factor.

SUMMARY

A typical dermatitis occurring in chicks fed purified rations adequate in pantothenic acid has been described. The properties of the protective factor agree with those reported for vitamin H and biotin and potent preparations of vitamin H have produced cures in 3 weeks when injected at a level of 35 μg. per day, the lowest level used.

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STUDIES ON THE THIAMIN REQUIREMENT OF YOUNG SWINE

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ONE TEXT FIGURE AND ONE PLATE (FOUR FIGURES)

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The present investigation on the thiamin (vitamin B_1) requirement of swine was suggested from observations on the association of dietary differences with growth and mortality of suckling pigs. Experimental work (unpublished) has demonstrated the value of skim milk as a supplemental feed to the regular ration of corn, mineral mixture and trinity mixture of tankage, 50 parts; alfalfa leaf meal, 25 parts; and linseed meal, 25 parts in the "creep" feeding of unweaned pigs. Losses from birth to weaning from various causes frequently amount to one-third of the total number of pigs farrowed. In addition, there is a considerable loss due to stunted growth and uneconomical gains. While most swine rations appear to contain liberal amounts of thiamin, the high requirement of the lactating female in general for this factor raises the question as to the nutritive state of the growing young. Accordingly, attention has been focused on pigs between the ages of 3 and 12 weeks when considerable food is required in addition to the dam's milk.

Since the present work was initiated, a number of reports, including those of Hughes ('38, '39), Chick and co-workers ('38), Wintrobe, Mitchell and Kolb ('38), Wintrobe ('39), and Davis, Freeman and Madsen ('40) have appeared on the role

of various members of the so-called vitamin B complex in the nutrition of swine. It now appears reasonably certain that thiamin, riboflavin, nicotinic acid, and perhaps several more additional factors are required.

One of the major problems in experimental studies on the requirements of swine for components of the vitamin B complex is that of a suitable basal diet. It is necessary that the diet be readily and economically prepared in quantity to enable the feeding of growing pigs for extended periods. While methods for study of thiamin appeared to be better established than for other factors, their application to the feeding of young pigs has remained in doubt.

The work to date has dealt with the response of pigs fed diets in which the thiamin was either destroyed by autoclaving or by sodium sulfite-sulfur dioxide treatment. A requirement for thiamin by young pigs has been demonstrated and a growth response and alleviation of a number of symptoms have followed supplementary feeding of graded amounts of crystalline thiamin hydrochloride to animals maintained on these deficient diets. Some of the physiological effects which have been encountered are similar to those described by the investigators already mentioned. Certain difficulties have been encountered in the ability to restore to and to maintain an optimum state of well-being by the feeding of thiamin along with rations treated to free them of vitamin B_1 by autoclaving. There has appeared to be a marked difference in the results obtained with the autoclaved diets and with the sulfite-sulfur dioxide treated diets, due perhaps to differences in destruction or inactivation of one or more other essential factors. In addition, other information on pathological changes in the organs and tissues has been gained which emphasizes the complexity of the problem.

EXPERIMENTAL PROCEDURE

The experimental work was conducted at the Beltsville Research Center, Beltsville, Maryland, between May, 1938 and February, 1940. In the preliminary experiments, the constitu-

ents of the diets which contained factors of the vitamin B complex were autoclaved in the usual manner and in the main series the same constituents were treated with sodium sulfite and sulfur dioxide to destroy thiamin according to the method described by Kline, Tolle and Nelson ('38). Besides the pig feeding work, parallel feeding experiments were conducted with rats which in a general way supported the findings on the pigs, although the symptoms seen in the swine are not comparable in all details to those observed in rats.

Pigs were obtained from the Bureau of Animal Industry herd at Beltsville, Maryland, at the age of 3 weeks and before they had begun to eat much feed other than the dam's milk. Not more than seven pigs were on experiment at any one time and each group represented a selection from either one or two litters. Most of the pigs were crossbred Poland China and Landrace while a few were purebred Poland China. The total number used during the 2 years was thirty-six. They were housed individually in metabolism cages on $\frac{1}{2}$ inch screen floors in a room kept at $75° \pm 2°$ F.

The composition of the sulfite-sulfur dioxide treated diets used in the main experiments is given in table 1. Alcohol- and acid-extracted casein was used throughout except for the control pig on diet CS5 in which case the casein was not treated. In diet CS7 untreated whey was used as a source of thiamin except for pig 2952 in which case additional pure thiamin was given. In diet CS5 the untreated whey and casein supplied the thiamin. It will be noted that the liver and mineral contents of diet S7 were increased over that of diet S5. The autoclaved diets were of similar composition to those in table 1. The liver and whey were autoclaved and dried in a vacuum shelf dryer. The lard was included at a level of 10% as representing an approximation to the fat intake of pigs being nursed by their dams but consuming considerable additional grain feed.

The animals were fed four times in each 24 hours for the first 3 weeks on experiment and thereafter three times a day. The basal diets were fed as a thin slop. The pigs were gener-

ally given a daily allowance of feed equivalent in weight to 4% of their body weight in terms of air dried feed, as long as their appetites were normal and thereafter lesser but regulated amounts suited to the rate of consumption. Effort was made to secure a uniform and regulated feed intake which could be recorded and compared with the thiamin intake and to the rates of growth. The fortified cod liver oil was listed as

TABLE 1

Percentage composition of the sulfite-sulfur dioxide treated diets

CONSTITUENTS	DIET NUMBER[1]		
	S5	S6	S7
Dried liver, SO_2 treated	5.0	8.0	7.5
Dried whey	15.0	15.0	15.0
Casein	25.0	25.0	25.0
Dextrin	20.0	20.0	27.5
Sucrose	21.0	18.0	9.5
Lard	10.0	10.0	10.0
Agar	2.0	2.0	2.0
Fortified cod liver oil	0.2	0.2	..[2]
Salt mixture	1.8	1.8	3.0
Calcium carbonate			0.5

[1] In the deficient diets, designated with the prefix D, the whey was treated with sulfite-sulfur dioxide and the casein extracted with water and alcohol. In the control diets designated with the prefix C, the whey was left untreated. The sulfite-sulfur dioxide treatment was applied for 8 days (in diets DS7 and CS7) instead of the usual 5-day period except in the case of pigs 3364 and 3366.

[2] In diet S7 the cod liver oil was fed at the level of 0.75 cc. per kilogram body weight per week.

containing 3000 U.S.P. units of vitamin A and 400 A.O.A.C. chick units of vitamin D per gram. Crystalline thiamin chloride hydrochloride abbreviated subsequently to "thiamin" dissolved in water was fed at different levels on a per-kilogram-body weight basis. It was added to the feed in the autoclaved series and fed by mouth three times per week in the sulfite series.

RESULTS

Experiments with pigs

In order to use nursling pigs in the feeding of diets composed largely of purified food substances, it was necessary

to determine whether normal health and growth could be maintained on control diets in which the thiamin and the other factors of the vitamin B complex were supplied in their natural state. In addition it was necessary to determine if the pigs could be reared in pens or cages equipped with screen floors. Control animals apparently met these requirements in a reasonably satisfactory manner. The pigs gained in weight, maintained their appetites and were essentially free of those symptoms and abnormalities which characterized the pigs fed treated diets and given either no or low levels of thiamin. The first twelve pigs used in the preliminary experiments with autoclaved materials suffered from other deficiencies than thiamin since, when ample thiamin was added, the animals did not grow at a normal rate and most of them developed convulsions and paralysis of the limbs accompanied by severe myelin degeneration of the cord and sciatic nerve. In two cases, liver concentrate appeared to be effective in curing or preventing these pathological symptoms. The animals also had severe scours and in some cases voided very dark colored urine. The convulsions were similar if not identical to the fits in swine described by Chick, Macree, Martin and Martin ('38) when their animals did not receive a chemically unidentified member (or members) of the vitamin B complex. The nerve degeneration observed was similar to that found by Ellis and Madsen ('40) in swine which developed incoordination when fed on certain natural feed combinations and heated diets and also to that described by Wintrobe, Mitchell and Kolb ('38). Since a thiamin deficiency uncomplicated by other deficiencies could not be obtained by autoclaving the vitamin B-carrying components of the diet, the change to sulfite-sulfur dioxide treatment was made.

Data on the young pigs used in the experiments with sulfite-sulfur dioxide treated diets are given in table 2 and in figure 1. Five animals received the deficient diets without regular feedings of thiamin and a sixth received such a small amount, namely, 6 μg. per kilogram body weight, as to be ineffective in prolonging the life of the animal. The histories of these

TABLE 2

Results on pigs fed diets treated with sulfite and sulfur dioxide

THIAMIN FED		PIG NO.	DIET NO.	DAYS ON EXPT.[1]	WEEKLY GAIN [2]	APPETITE	VOMITING	NEAR DEATH OR END EXPT.		AUTOPSY		
Per kg. body wt.	Day started							Leg deformity	Rectal temp.	Heart	Alimentary tract	Body condition
µg.					*kg.*				*° F.*			
0	—	2031	DS5	D. 28	Loss	Poor	Some	None	—	Flabby	Erosions	Emac.
0	—	2586	DS5	M. 33	Loss	Poor	Frequent	None	99.4	Flabby	Normal	Emac.
Two doses[4]	28–58	2705	DS5	M. 133	Varied	Varied	Some	None	93.5	Flabby	Normal	Emac.
0	—	2957	DS7	D. 34	Loss	Poor	Frequent	None	96.0	Pale	Congested	Emac.
0	—	3368	DS7	D. 39	Loss	Poor	Frequent	None	99.3	Mottled	Congested	Emac.
6	21	2958	DS7	D. 34	Loss	Poor	Frequent	None	100.3	Normal	Congested	Thin
15	15	3364	DS7	D. 36	2.1	Fair	Some	None	102.8	Pale	Normal	Good
15	15	3363	DS7	E. 85	1.4	Fair	None	Slight	102.4	Flabby	Normal	Fair
25	1	2032	DS5	E. 94	2.3	Good	None	Marked	—	Normal	Normal	Good
25	1	2587	DS5	E. 113	2.7	Good	None	Moderate	103.0	Normal	Normal	Good
25	15	3366	DS7	E. 85	2.5	Good	None	Moderate	103.2	Normal	Normal	Good
25	15	3365	DS7	E. 85	2.2	Good	None	Slight	102.6	Normal	Normal	Good
12 to 38	18	2959	DS7	E. 125	3.4	Varied	None	None	102.8	Normal	Normal	Good
18 to 38	18	2960	DS7	E. 125	3.3	Varied	None	None	103.8	Normal	Normal	Good
25 to 50	18	2951	DS7	E. 97	2.6	Varied	Slight	None	102.3	Normal	Normal	Fair
50	1	2033	DS5	E. 94	2.3	Good	None	Marked	—	Normal	Normal	Good
50	1	2706	DS5	E. 99	2.4	Good	None	Moderate	102.7	Normal	Normal	Good
50	1	2062	DS6	E. 94	2.5	Good	None	Marked	—	Normal	Normal	Good
50	15	3367	DS7	E. 85	3.4	Good	None	Moderate	103.4	Normal	Normal	Good
100	1	2034	DS5	D. 80	2.3	Good	None	Slight	—	Normal	Congested	Fair
100	1	2707	DS5	E. 99	1.1	—	None	Severe	103.4	Normal	Normal	Thin
Control	—	2035	CS5	E. 94	4.2	Excellent	None	None	—	Normal	Normal	Good
Control	—	2952	CS7	E. 97	2.1[3]	Good	None	None	102.8	Normal	Normal	Good
Control	—	3362	CS7	D. 41	1.9	Good	None	None	100.4	Normal	Congested	Good

[1] D. indicates death; M. moribund condition and E. that animal survived the experiment in fair or normal condition.

[2] In the case of the animals fed thiamin, the weekly gain is calculated for the period during which a designated level was fed. For animals 2951, 2959, and 2960 the gains for the last 28 days during which the maximum levels of thiamin were fed were used in the calculation.

[3] Food allowance was limited to a 3% ration for first half of the experiment.

[4] Each dose contained 1.8 mg.

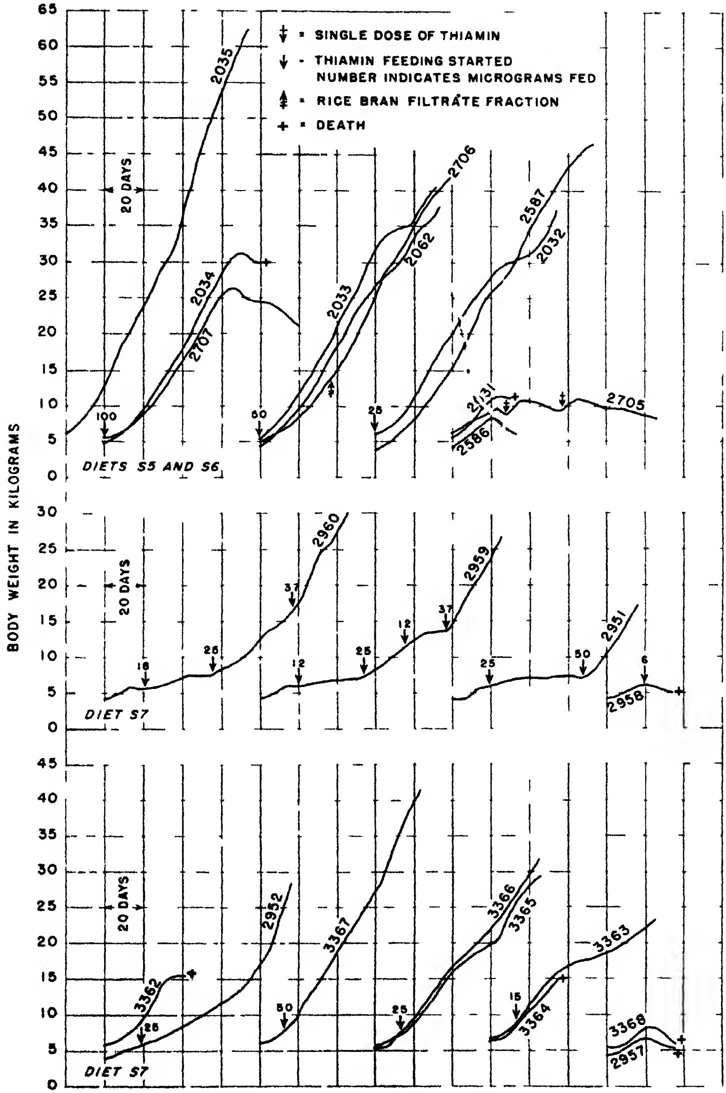

Fig. 1 Growth of pigs fed diets of the sulfite sulfur dioxide series. The level of thiamin fed per kilogram body weight is indicated by the number (in micrograms) over the arrows pointing downward. Pigs 2035, 2952, and 3362 were positive controls. The remainder were on the deficient diets. Pig 2952 was restricted in food intake during the first part of the experiment.

pigs were characterized, as in the autoclaved series, by decreases in appetite and in body weight after the initial 3-week depletion period. Frequent vomitings were also noted.

Rectal temperature readings showed a marked decline from normal for several days preceding death. Four animals showed congested areas in the stomach or intestines. These same animals gave evidence of cyanosis at the time of or shortly before death. Pig 2031 died unexpectedly and upon autopsy considerable straw-colored fluid was found in the thoracic and pericardial cavities. Besides being flabby, the heart was paler than normal and the cardiac muscle was streaked with gray tissue particularly in the wall of the left ventricle. There was considerable serous atrophy of the cardiac fat. Histological examination revealed scattered areas of atrophy and necrosis of heart muscle fibers, a condition suggestive of infarction. On gross examination the liver appeared congested. Sections of liver tissue showed widespread central necrosis of the lobules with congestion around the periphery of the lobule. The lesions of the alimentary tract noted in table 2 were confined largely to the gastric mucosa in the pyloric region of the stomach.

Degenerative changes were found in the cardiac muscle of the walls of the left ventricle and of the inter-ventricular septum and in the liver of pig 2586 which was killed when in a moribund condition.

Pig 2705 showed the usual decline in weight during the fourth week on experiment and was then given a total of 1.8 mg. of thiamin over a 4-day period. There was an immediate response in increased appetite, gain in weight and rise in body temperature. By the end of 8 weeks the animal was again listless, without appetite and subnormal in body temperature. A second dose of 1.8 mg. of thiamin produced results identical to those in the previous instance. The body weight increase was approximately 2 kg. each time and was reached in 7 days. Following this final period of weight increase, the animal was kept on experiment for 6 weeks longer. The body temperature declined to 93.5° F. On the one hundred and thirty-third day

of the experiment, the animal was in a moribund condition and was killed. Extreme emaciation was the outstanding characteristic of the autopsy findings. As in several other cases, the heart appeared flabby. Histological examination of the heart muscle showed degenerative changes. Unlike other pigs, which received no regular thiamin therapy, the alimentary tract was apparently normal on gross examination.

Autopsy examinations on pigs 2957, 2958, and 3368 gave results similar to those just discussed. The changes in the heart tissues of these animals were especially noteworthy. The average time of survival on the thiamin-deficient diets for the five pigs was 35 days.

The feeding of thiamin gave favorable responses in the usual case. From a level of 15 to 50 μg. per kilogram body weight per day, there was evidence of a general improvement in rate of growth, appetite, and freedom from those abnormalities in organs and tissues noted among the animals on the deficient diets. On diets DS5 and DS6, good growth was obtained for 6 to 8 weeks on 25 μg. just as on 50 and 100 μg. of thiamin when no depletion period was allowed. Later, however, lameness developed among the animals. The symptoms resembled those of rachitic animals in contrast to the locomotor incoordination among pigs fed the autoclaved diets. Undoubtedly, confinement on the screen floors may have accentuated the stiffness and awkwardness in movement. Several of the animals receiving thiamin showed decreased appetite and rate of growth along with development of lameness. However, no convulsions developed, likewise in contrast to the results on the autoclaved diets. At one time during the feeding period all of the pigs of the 2032–2062 series developed acute diarrhea. Pig 2034 died presumably as a result of the attack. The chief findings at autopsy were congestion of the lungs and occasional areas of congestion in the small intestine. The diarrhea cleared up in the other animals in about a week. The difficulties with lameness and other evidence of dietary deficiencies among the animals just described prompted the feeding of a filtrate fraction of rice bran to pig 2706 from the

thirty-seventh to the seventieth day of the feeding period. As shown in table 2 and figure 1, this animal remained in good health except for a moderate degree of leg deformity. However, its condition was no different from that of other animals which did not receive the rice bran supplement, as for example no. 2587. Pig 2707 became very lame and was unable to stand upon its feet, even to eat and drink.

All the surviving animals fed on diets DS5 and DS6 were slaughtered after they had been on their experimental diets for 14 to 16 weeks, and no abnormal pathology was seen in the organs or intestinal tract. However, these animals as a group tended to have enlarged leg joints; bones appeared slightly softer than usual and the spinal column of some of the animals appeared more curved than normal. The serum calcium of the pigs on the treated diets was uniformly low and the inorganic phosphorus and the phosphatase values high. No abnormalities were noted in blood sugar, non-protein nitrogen, hematocrit or red and white blood cell counts. Bone analyses indicated an abnormally low ash content for the deficient pigs. These findings suggest a possible interference with mineral metabolism in the animals fed diets DS5 and DS6. A histological examination of the sciatic nerve and spinal cord of these animals also revealed some changes which indicate that the diets were probably deficient in other factors besides thiamin which was specifically studied in this series of experiments.

The pigs fed diets DS5 and DS6 showed a satisfactory response to thiamin treatment except for the rachitic-like lameness symptoms apparently resulting from deranged mineral metabolism and to a slight extent from mild nerve degeneration. Since calcium, phosphorus, and cod liver oil levels were suspected as playing a part, these nutrients were increased in diet S7 which was fed to the rest of the pigs. The cod liver oil was fed separately at the level of 0.75 cc. per kilogram of body weight per week. Although diets DS5 and DS6 were found to contain approximately 0.42% calcium and 0.56% phosphorus which is within recognized requirement standards, the calcium content was doubled and the phosphorus content

increased by 20% in the new diet. Another modification introduced in diet DS7 for the feeding of a number of pigs was the 8-day sulfite-sulfur dioxide treatment of the liver and whey. This was done because of the suggestion from both rat and pig feeding experiments that a 5-day treatment was insufficient to destroy all the thiamin. Pigs numbered from 2951 and above were fed the S7 diet.

The earlier work suggested that 25 µg. of thiamin daily per kilogram of body weight was possibly adequate for growth. Accordingly, thiamin was fed at 6, 13, 19, and 25 µg. levels, starting after 21 days on the deficient diet alone (fig. 1). Feed intake was restricted at first to 3% of body weight with some animals refusing to eat even this allowance. All levels of thiamin proved too low for promotion of normal growth. As indicated in figure 1, changes were made in the thiamin levels of pigs 2951, 2952, and 2960. It was found that a 37 µg. level resulted in better growth than 25 µg. and essentially as good as 50 µg. These three animals, like no. 2952 on the control diet, remained free of abnormal symptoms except for instances of loss of appetite, vomiting, and low body temperature alleviated by increased thiamin dosage.

The final group of seven pigs, all from one litter and numbered from 3362 to 3368, confirmed the results already obtained. Pigs 3365 and 3366 were fed 25 µg. of thiamin and gained 2.2 and 2.5 kg. per week respectively, as compared to pig 3367 which gained 3.4 kg. per week on 50 µg. of thiamin. Pigs 3362 and 3364 died before the conclusion of the test. Autopsy examination of pig 3362 fed diet CS7 showed considerable congestion in the intestinal tract, and, like pig 3364, which was given the 5-day treated diet and 15 µg. of thiamin showed an accumulation of free fluid in the body cavities. Other than these findings together with some diarrhea preceding death, the exact causes of death could not be determined. Unlike the pigs fed on diets DS5 and DS6, those on diet DS7 showed normal calcium, phosphorus and phosphatase values. However, locomotor abnormalities persisted to a slight degree.

Experiments with rats

Some results from rat feeding trials on the same diets as fed to the pigs are shown in table 3. The average weekly weight changes calculated for the last 5 weeks of the 7-week test period and the animal survival figures generally confirm the results obtained on the pigs. The rats which received no thiamin usually developed symptoms of polyneuritis. They were left on the test diet in the usual case until death. Rats on similar autoclaved diets did not grow as rapidly as those shown in table 3 when thiamin was fed as a supplement.

TABLE 3

Results on male rats fed the deficient diets during a 7-week period with and without added thiamin during the last 5 weeks

DIET	NO THIAMIN			60 µg. THIAMIN WEEKLY	
	Number of rats	Number alive after 7 weeks	Weekly loss in weight [1]	Number of rats	Weekly gain in weight [1]
			gm.		gm.
DS5	7	7	—1.1	6	18.7
DS7 (5 day)	7	3	—2.1	6	24.4
DS7 (8 day)	7	3	—4.2	6	23.3

[1] Figures based on changes in weight from the third to the seventh week inclusive.

The difference between the growth rate of rats on diet DS5 as compared with DS7 when thiamin was fed as a supplement was probably due to the increased percentage of liver in the latter diet. Treatment of the whey and liver for 8 days with sulfite and sulfur dioxide as compared to 5-day treatment was reflected in the weekly weight changes of the rats receiving no supplemental thiamin. More recent work (unpublished data) indicates however that with careful regulation of the pH of the sulfite-sulfur dioxide treating medium as complete a destruction of thiamin occurs when treated 5 days as when treated 8 days. It is recognized that small and probably variable amounts of thiamin may have been present in the basal diets which were not detected either in the pig or rat feeding work.

DISCUSSION

When whey and liver were autoclaved and vacuum dried, destruction of other vitamins than thiamin undoubtedly oc-

curred. On the other hand the treatment of whey and liver with sulfite and sulfur dioxide especially as used in diet DS7 seemed to be specific for the destruction of thiamin since the addition of thiamin alone enabled the pigs to grow at approximately a normal rate without developing other complicating deficiency symptoms to any marked extent.

The symptoms observed in the pigs on the autoclaved diets are worthy of note as they were probably caused by the destruction of other unknown factors necessary for the adequate nutrition of the pig. The myelin degeneration seen in the pigs on these experimental diets appears to have been due to other deficiencies than thiamin. This view is in accord with results obtained by Eveleth and Biester ('37), Wintrobe, Mitchell and Kolb ('38) and Ellis and Madsen ('40), and by other investigators using other animals than the pig.

The results obtained on diet DS5 and DS6 were complicated by inadequate mineral nutrition. This defect was largely corrected in diet DS7. It was of interest to note that pig 2035 on diet CS5 did not become lame nor was his serum calcium low. This would indicate that possibly the sulfite-sulfur dioxide treatment of the whey in some manner rendered the minerals of the whey unavailable to the pig.

An approximation of the vitamin B_1 requirement of young pigs appears to be indicated especially from the results obtained with animals on diet DS7. Apparently over the period of growth studied in these pigs the requirement per kilogram body weight is more than 25 μg. per kilogram per day, while a level of 37 and 50 μg. appears to be enough thiamin to maintain an approximately normal rate of growth. Cowgill's formula ('34) when applied to these small nursling pigs gives values much lower than the values obtained above; in applying his formula the maximum weight of the species was taken to be 380 kg. (see Russell, '22).

Although the animals were fed thiamin on a body-weight basis, the regulation of feed intake to the same basis and the keeping of approximate feed consumption records also permit the expression of thiamin intake on the basis of total feed

and of the sum of protein and carbohydrate. Thus a 4% feed allowance provides 40 gm. per kilogram body weight daily; and where the thiamin allowance was 37 µg. the thiamin intake per 100 gm. of feed was approximately 94 µg. Tabulation of actual data is given in table 4 for those pigs successfully reared on diet DS7. As would be expected the data in column 6 show an increase in thiamin intake per 100 gm. of feed as the thiamin allowance per kilogram body weight increased. It is interesting to note that in the first four cases where the thiamin intake ranged from 47 to 70 µg. the pigs actually maintained their body weight or even gained.

TABLE 4

Approximate thiamin intake per 100 gm. of feed consumed for pigs on diet DS7

PIG NO.	THIAMIN LEVEL PER KILOGRAM BODY WT.	DAYS FED ON GIVEN THIAMIN LEVEL	TOTAL APPROXIMATE FEED CONSUMED	TOTAL MILLIGRAMS THIAMIN CONSUMED	APPROXIMATE THIAMIN INTAKE PER 100 GM. OF FEED
	µg.		*kg.*	*mg.*	*µg.*
2959 [1]	12	37	6.51	3.05	47
2959 [1]	12	21	6.48	3.29	51
3363 [1]	15	71	29.56	16.94	57
2960 [1]	18	35	6.00	4.17	70
2959 [1]	25	21	5.47	4.58	84
2960 [1]	25	44	15.76	11.97	76
2951 [1]	25	49	9.11	8.31	91
3365 [1]	25	71	38.18	29.58	77
3366 [1]	25	71	43.43	31.20	72
2959	37	28	20.22	18.41	91
2960	37	26	21.80	21.45	98
2951	50	28	10.20	14.09	138
3367	50	71	57.34	74.91	131

[1] These animals limited their rate of food consumption presumably because not enough thiamin was fed. The remaining pigs ate all the food placed before them.

If an average is taken of the five values obtained for those pigs receiving thiamin at the 25 µg. level a value of 80 µg. per 100 gm. of air dry feed is obtained. An average of 94 µg. is obtained for those two pigs fed the next highest or 37 µg. level of thiamin fed. Since the pigs on the 25 µg. level refused some feed while those on the 37 µg. level ate all the feed placed before them the actual thiamin requirement appears to be be-

tween these two levels or between 80 and 94 µg. per 100 gm. of feed.

Assuming the diet to contain approximately 75% of carbohydrate and protein the requirement per 100 gm. of carbohydrate and protein can be placed at between 106 and 125 µg.

The above figures are in fairly close agreement with those given by Arnold and Elvehjem ('39) for the thiamin requirement of the dog, Jukes and Heitman ('40) for the thiamin requirement of the chicken, and the value indicated for all species by Williams and Spies ('38). These approximations of the thiamin requirement of pigs hitherto unreported, may be considered as another piece of evidence that the thiamin requirement for all species of animals may be the same when expressed per unit of carbohydrate and protein consumed.

SUMMARY

Thirty-six pigs were fed autoclaved and sodium-sulfite sulfur dioxide treated basal diets with and without a supplement of thiamin. In the preliminary work on the autoclaved diets, animals developed convulsions, locomotor incoordination, paralysis, and other symptoms of malnutrition even when thiamin was given.

Animals on the sodium sulfite-sulfur dioxide treated diets without added thiamin consistently developed symptoms due to a specific lack of thiamin. These symptoms were: almost complete refusal of food with occasional vomiting, extreme emaciation, and marked lowering of body temperature. When thiamin was not added, death usually occurred within 5 weeks. Upon autopsy of these animals a very flabby heart was noted. The intestinal tract was always almost completely empty but pathological changes were not noted in every case. In some cases liver damage was noted.

When enough thiamin was added, an approximately normal rate of growth was maintained with the alleviation of all deficiency symptoms. Attempt has been made to relate data on thiamin requirements to feed intake as well as to body weight. For swine under the experimental conditions described the

supplemental thiamin chloride hydrochloride requirement appears to be between 106 and 120 µg. per 100 gm. of carbohydrate and protein consumed.

Rats fed the same diets as those fed the pigs substantiated the results obtained with the pigs.

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PLATE

PLATE 1

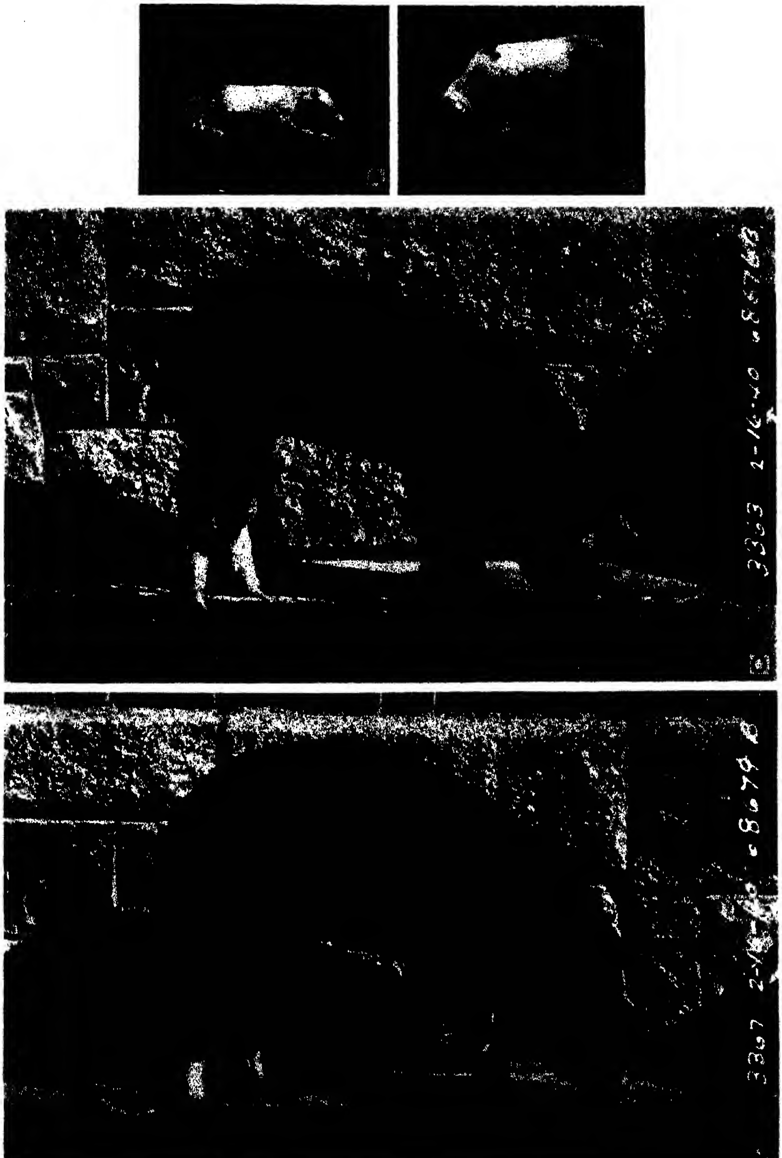
EXPLANATION OF FIGURES

A Pig 2586. Picture taken at 56 days of age after being on the thiamin deficient ration DS5 for 32 days. The animal died from thiamin deficiency the next day after the above picture was taken. Weight 6.07 kg.

B Pig 2587. Litter mate to pig 2586. Picture taken at the same time as that of pig 2586. Weight 14.64 kg. The only difference in the treatment of the two animals was the feeding of 25 μg. of thiamin per kilogram body weight to pig 2587 during the 32-day period preceding the taking of the picture.

C Pig 3363. Picture taken at 99 days of age. This animal had received 15 μg. of thiamin per kilogram body weight for 64 days after a thiamin depletion period of 14 days on diet DS7. Weight, 21.79 kg.

D Pig 3367. Litter mate to pig 3363. Picture taken at the same time as that of pig 3363. Weight 37.8 kg. Pig 3367 received 50 μg. of thiamin per kilogram of body weight as compared to 15 μg. fed pig 3363.



ANTI-GREY HAIR VITAMIN DEFICIENCY IN THE SILVER FOX [1]

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THREE FIGURES

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It has been found in this and other laboratories that rats, dogs and guinea pigs (Morgan and Simms, '40) suffer extensive depigmentation of the fur as a result of "filtrate factor" or anti-grey hair vitamin deficiency. When the opportunity [2] to produce a similar deficiency in young silver foxes was offered us we accepted it with interest because of the peculiar importance and variety of fur color in this species. Normally the markings on silver fox fur resemble those of black rats during the earlier stages of the anti-grey vitamin deficiency. It seemed possible that this species might be especially susceptible to the greying deficiency. After our experiment had been begun a somewhat similar study was reported by Lunde and Kringstad ('39). They gave an alkaline autoclaved yeast extract and synthetic vitamin B_1 along with a vitamin B-free basal diet to foxes 11 weeks of age. After 8 to 10 weeks, greying began on the snouts and worked backward toward the eye-sockets. Control animals given the anti-grey substance which they call B_x were of normal color. They mention the work of

[1] This investigation has been aided by a grant from the Josiah Macy, Jr. Foundation. Assistance was rendered also by Works Progress Administration Official Project 665-08-3-30, Unit A-24, assigned to the University of California.

[2] This experiment was made possible through the cooperation of Mr. F. J. Mullins of East Bay Fisheries, Inc. of San Francisco and Mr. Elmer Atkinson, fox breeder of Aptos, California. We gratefully acknowledge their indispensable assistance.

Schoop ('38) who described a "sand coloredness' in newborn foxes which he thought due to lack of vitamin C. Lunde and Kringstad state, however, that they had found the fox not to require vitamin C, an observation confirmed by our experiment. Lunde and Kringstad concluded that the "sand coloredness" must have been caused by anti-grey vitamin deficiency.

EXPERIMENTAL

Six young silver foxes from three litters of highly inbred stock were used in our experiment. They were kept in raised open pens on chicken wire and were never allowed to run in

TABLE 1

Growth of foxes on purified diet

FOX, NUMBER AND SEX	LITTER NUMBER	DIET	WEIGHTS IN KILOGRAMS June 12	July 8	July 22	Sept. 5	Nov. 20	Dec. 11
1, ♀	3	Normal	1.86	2.78	3.17		3.91	4.14
2, ♂	1	Normal	2.53	3.58	4.43		5.40	5.55
3, ♂	2	Normal	2.30	3.83	4.06		5.98	6.18
4, ♀	2	Deficient	2.02	3.13	3.30	3.98	4.40	4.38
5, ♂	1	Deficient	2.41	3.47	3.63	5.45	6.42	6.55
6, ♀	2	Deficient	2.10	2.95	3.24			

the open or to touch the soil. The parents were each fed 12 to 14 ounces daily of a breeding ration made up of 30% fresh meat products, 30% canned whole fish,[3] 40% dry cereal fox feed, and in addition 2 to 3 ounces of carrots and figs. The young were born late in April, 1939 and were weaned the last week in May. The growth of the six animals and the diets to which they were assigned are indicated in table 1.

The basal diet was a modification of that used for dogs in a similar experiment. Its composition, in per cent, was washed casein [4] 30, hydrogenated cottonseed oil [5] 10, salt mixture 2.5,

[3] Silvertone, canned California sardines packed with concentrated fish juices by East Bay Fisheries, Inc.

[4] The washed casein was kindly supplied us by Dr. Paul D. V. Manning of the Western Condensing Company of San Francisco.

[5] Crisco.

wheat germ 15, sucrose 39.5, bone ash 2, agar 1. The wheat germ provided 3.75 mg. thiamin per kilogram of diet and undoubtedly sufficient vitamin B_6. There were added 35 mg. nicotinic acid and 2 mg. riboflavin per kilogram of diet, and each fox was given separately daily 1 teaspoonful (4 gm.) of soup-fin shark liver oil [6] containing 6000 units of vitamin A and 420 units of vitamin D per gram. For three of the animals, nos. 1, 2, and 3, the basal diet was supplemented with 19 cc. per kilogram of yeast filtrate which was known to be rich in the anti-grey hair factor. By actual test with rats each cubic centimeter of the filtrate preparation was found to be equivalent to 1.2 gm. of the yeast from which it was made, thus providing the diet with filtrate potency equivalent to 2% of dried yeast in the ration.

RESULTS

The regular stock ration was gradually replaced by the purified diet over a period of 3 to 4 weeks until by July 1 the animals were receiving practically only the latter. By July 18 the three foxes on deficient diet were rapidly losing fur so that their coats appeared short and cottony like those of very young animals. Their snouts and ears became grey in color. The male, no. 5, developed a dry scaly condition with loss of hair on the tail. On July 26 fox no. 6, which had been active and had maintained food intake as well as any of the others, suddenly died. On the same day an autopsy was performed and tissues saved for histological examination. No evidence of infection or any other abnormality was found except for the presence of a greatly enlarged and hemorrhagic thymus.

Both lots of foxes had eaten the basal diet somewhat unenthusiastically but those on the deficient ration ate considerably less than the others. Beginning August 3 the two surviving deficient animals were given the regular stock ration, 8 to 11 ounces, plus 1 to 3 ounces of the basal diet. Nevertheless they continued to lose fur until the tails were hairless and the skin

[6] Supplied to us by East Bay Fisheries, Inc.

was hard and dry. Beginning August 20 a concentrated rice bran preparation was given, 1 teaspoonful daily per fox, and within 2 weeks a new growth of creamy under-fur began to appear. This under-fur developed rapidly, darkened somewhat but retained an unusual silvery grey color. The guard hairs, particularly on the tails, were slow in appearing and somewhat brownish in color.

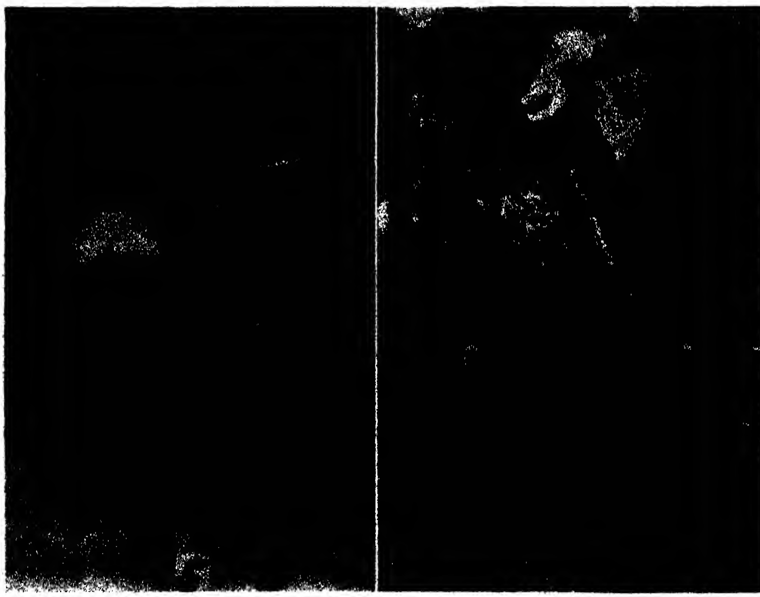

Fig. 1 Photographs of foxes fed experimental diets taken during the fur re-growth period in October, 1939. Fox 5 had suffered the anti-grey vitamin deficiency for 44 days, fox 3 had adequate diet.

In the meantime the three animals which had received the filtrate factor-containing diet made excellent growth and did not lose fur at any time. Their under-fur had the usual very dark, almost black, color and the guard hairs were long and dark with somewhat less than the expected silvering. The photographs shown in figure 1 were taken in October.

On December 11, 1939, all five remaining foxes were killed with carbon monoxide gas and pelted. Careful autopsies [7] were made in all cases and tissue specimens saved for study. Thyroids, kidneys, livers, lungs, heart, spleen and sex glands were weighed but no divergence from normal was seen in any of these organs. The only abnormality noted was the presence

Fig. 2 Front view of pelts of the surviving foxes. Numbers 4 and 5 had anti-grey vitamin deficient diet for 44 days, nos. 1, 2 and 3 had received an adequate diet.

of large red mottled thymuses in the two deficient animals and no trace of persistent thymus in the other three.

Microscopic examination [8] of all the organs revealed no abnormality in any of the hearts, spleens, thyroids, stomachs,

[7] We wish to thank Nobuko Shimotori and Mary Groody who assisted in the autopsies.

[8] We acknowledge the invaluable assistance of Dr. D. A. Singman of the Pathology Division, University of California Medical School, in the study of these tissues.

testes or ovaries. Mild passive congestion was found in the lungs and mild or moderate fatty infiltration in livers and in renal tubules of all the animals killed with carbon monoxide. These changes are to be expected in asphyxia. No evidences of infection were found. The surviving thymuses of the three

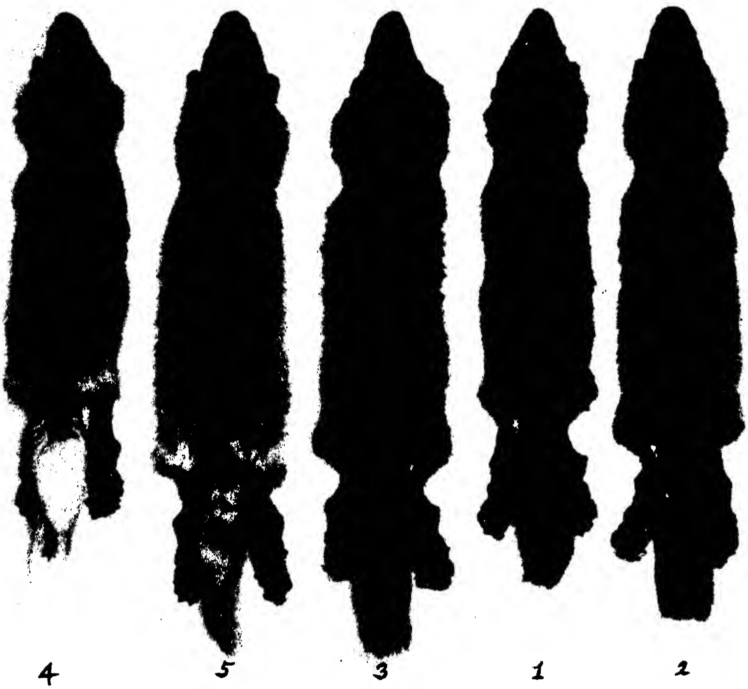

Fig. 3 Back view of pelts of surviving foxes.

deficient animals were mottled with surface hemorrhages, that of no. 6, which died early more definitely than the others. None of them showed any evidence of involution.

Slight abnormality of the adrenals occurred in the deficient animals, some deeply pigmented cells being present in the medulla and vascular and partly degenerated areas in the reticularis at the polar ends of the cortex next to the medulla,

but the condition was not as pronounced as that we have seen in numerous rats which had become grey from the same deficiency.

The pelts were photographed as soon as possible after their removal and are shown in figures 2 and 3. Fox no. 4 was most depigmented with completely grey brush and both 4 and 5 had white belly fur, a phenomenon not usually seen even in the lightest colored silver foxes.

DISCUSSION

The Norway platinum foxes recently obtained as mutants as described by Mohr and Tuff ('39) have distinct white markings on the face, legs, neck and belly as well as only partly pigmented under-fur and guard hairs. These markings appear to be inherited as a dominant characteristic in heterozygous individuals. Whether long-continued slight anti-grey vitamin deficiency may be involved in the production of such mutants is an interesting question. Certainly these silver foxes were more susceptible to the depigmenting deficiency than the rats and dogs previously studied in this laboratory.

The most outstanding feature of this experiment was the early and devastating effect upon the fur of the deprivation of filtrate factors even though this deprivation was only partial. The diet contained 15% of whole wheat germ which was included chiefly to provide vitamin E but which was by no means devoid of the filtrate factors. Nevertheless after only 26 days on this regime one of the foxes died apparently of the deficiency, and although the two remaining animals were changed at once to the complete stock diet and were given additional doses of rice bran concentrate their fur was lost and the new fur which appeared was largely depigmented. The survival of thymus which was red-pigmented in spots and without evidence of involution in each of the three animals on the deficient diet is another important observation. This thymus survival may be connected with the slight but unmistakable damage to the adrenal cortex seen in the same animals. It has been shown by Crede and Moon ('40) that adrenocorticotropic extracts exert

a thymus destructive effect in normal and castrate but not in adrenalectomized rats. The adrenal cortical secretion would therefore appear to be at least partially responsible for the normal disappearance of the thymus. Abnormal survival of the thymus noted in the experimental foxes may perhaps be ascribed to partial failure of adrenal cortex influence.

McKibbin, Madden, Black and Elvehjem ('39) found "a peculiar red pigment spotted appearance of the thymus gland" in two dogs which had died after relatively short periods on filtrate-factor deficient diet. The ages of these dogs were not given but apparently they were young, supposedly 2 to 3 months old if they were placed on the diet at weaning. They lived only 20 and 30 days after the supplementary feeding began, a much shorter survival than we have usually found with filtrate-factor deficient dogs. Fouts, Helmer and Lepkovsky ('40) who used adult dogs likewise noted longer survival periods. Thymus survival might be expected in dogs of the age mentioned but apparently our normal foxes nos. 1, 2 and 3, 7 to 8 months of age, had lost so much of the thymus as to make it impossible to find the remnants although they were carefully sought.

The question arises as to whether decreased adrenal cortex activity was the primary result of the filtrate factor deficiency with hypertrophied thymus resulting therefrom as noted in Addison's disease (Rowntree, '35), or whether the thymus is primarily affected. There may be in this a clue to the mysterious role of the thymus and the clinical condition known as "status thymicolymphaticus."

SUMMARY

Six young silver foxes of three litters were placed on purified diet supplemented by fish liver oil, wheat germ, riboflavin and nicotinic acid, three receiving also a yeast "filtrate factor" preparation. One of those which did not receive filtrate factor died after 26 days and the other two had lost most of their fur although they had been left on the deficient diet only 44 days. Their new fur was light grey in color.

On autopsy of the fox that died and the other two on deficient diet pelted 4 months later large red-mottled thymuses were found.

The three foxes which received the filtrate factor retained their fur, had no surviving thymuses and had normal dark silver pelts.

These observations are similar to those previously observed in rats and dogs and point to possible interrelation of nutritional and endocrine mechanisms.

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MEAD JOHNSON & COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1941 Award of $1,000 established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute at Chicago on April 16, 1941.

The Award will be given to the laboratory (non-clinical) or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year January 1st to December 31st the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the prize be divided between two or more parties. It may also be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1940 must be in the hands of the Secretary by January 25, 1941. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

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ERRATUM

In table 1, page 519 of the paper "Fasting catabolism and food utilization of calcium-deficient rats," by Max Kleiber, Muriel D. D. Boelter and David M. Greenberg, which appeared in The Journal of Nutrition, vol. 19, no. 6, June, 1940, the synthetic vitamin supplements should be per kilo of diet; namely Thiamin Chloride 5.0 mg. synthetic riboflavin 4.0 mg., and nicotinic acid 50.0 mg. per *kilo of diet*.